UNIVERSITY OF ALBERTA

MODULATION OF ARYL HYDROCARBON RECEPTOR-REGULATED GENES BY ARSENITE, CADMIUM, AND CHROMIUM.

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

IN

PHARMACEUTICAL SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

Spring 2008

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Your file Votre référence
ISBN: 978-0-494-45420-6
Our file Notre référence
ISBN: 978-0-494-45420-6

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ABSTRACT

Aryl hydrocarbon receptor (AhR) ligands and heavy metals are environmental co-contaminants and their molecular interaction may disrupt the coordinated regulation of AhR-dependent phase I and II drug metabolizing enzymes. As such, the effect of the heavy metals, As^{3+} , Cd^{2+}, and Cr^{6+}, on the AhR-regulated genes: cytochrome P450 1a1 (Cyp1a1), NAD(P)H: quinone oxidoreductase (Nqo1), and glutathione transferase a (Gst a) was investigated in murine hepatoma Hepa 1c1c7 cells. All three metals inhibited the induction of Cyp1a1 activity by AhR ligands while increasing its mRNA expression. On the other hand, As^{3+} and Cd^{2+} enhanced Nqo1 and Gst a at the activity and mRNA levels while Cr^{6+} inhibited their constitutive and inducible expression. Studies investigating the mechanisms involved in the modulation of these AhR-regulated genes revealed a AhR-dependent induction of *Cyp1a1* transcription. On the other hand, heavy metals alter Nqo1 expression at the transcriptional level, through a labile protein-mediated pathway. Transcriptional regulation was responsible for the up-regulation of Nqo1 activity by As^{3+} and Cd^{2+}, whereas post-translational mechanism(s) were involved in the inhibition of Cyp1a1 activity by all three metals. Using the prooxidant buthionine-(S,R)-sulfoximine (BSO) and the antioxidant N-acetylcysteine (NAC), we were able to demonstrate that As^{3+}-, Cd^{2+}-, and Cr^{6+}-induced oxidative stress is responsible for the modulation of Cyp1a1 at the transcriptional and posttranscriptional levels and the induction of Nqo1 and Gst a at the transcriptional level. These results show that the ability of metals to alter the capacity of AhR ligands to induce the bioactivating phase I and the detoxifying phase II enzymes will influence the carcinogenicity and mutagenicity of the AhR ligands. Thus, it comes at no surprise that As^{3+} demonstrates anticancer effects. It is likely that the

therapeutic efficacy of As^{3+} is associated with an increase in Nqo1 and Gst a since ascorbic acid potentiates the anticancer effect of As^{3+} while enhancing the expression of HO-1, Nqo1, and Gst a. Future studies are required to determine the effect of heavy metals on the metabolism of AhR ligands to quantify their effect on the mutagenicity of environmental pollutants and determine the exact role of AhR-regulated genes in the initiation and progression of malignancies.

Dedicated to

My father, my inspiration
My mother, my guidance
My husband, my foundation

ACKNOWLEDGEMENTS

This work would not have been completed without the support and encouragement of a magnificent group of people.

Dr. Ayman El-Kadi. I am indebted first and foremost to my supervisor for his trust, encouragement, unconditional support, and invaluable guidance. Thank you for believing in me more than I did in myself.

Hussein Elbekai. My late father who believed in me, as a 12-year old girl. He provided me with a pillar in my life, lighted my path, and drew my future. His life gave me the inspiration and drive, and his soul gave me the strength and patience. I wish you were here.

Ihsan Elbekai. My mother, who is more deservant of a PhD than me. I am the person I am today because of her hardwork, dedication, and the strongest initiative one could see in a woman.

Aktham Zahalka. My husband: my unfailing source of love, support, encouragement and patience. Your gift of inner peace is more than I deserve.

All of my amazing sisters and terrific brother. Their love and support is surreal and means the world to me.

My khalto and uncle, my incredible second set of parents.

I sincerely acknowledge the members of my supervisory committee, Dr. John Seubert and Dr. Chris Le for their continued encouragement, help and support. I would also like to thank the rest of the academic and non-academic staff at the Faculty of Pharmacy for their generous help and support.

I would like to thank my lab colleagues for the great friendships during 5 years of graduate work.

Finally, I am grateful for the generous financial support provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) as they supported me with the Canada Graduate Scholarship-Doctoral (CGS-D). I am also indebted to the University of Alberta for supporting me with the University of Alberta PhD Scholarship, the Izaak Walton Killam Memorial Scholarship, and others.

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LIST OF ABBREVIATIONS

3MC	3-Methylcholanthrene
AA	Arachidonic acid
AhRE	Ah-responsive element
Act-D	Actinomycin D
AhR	Aryl hydrocarbon receptor
ALL	Acute lymphocytic leukemia
AML	Acute myelogenous leukemia
APL	Acute promyelocytic leukemia
AP-1	Activator protein-1
ARE	antioxidant-response element
ARNT	aryl hydrocarbon receptor nuclear translocator
As^{3+}	Arsenite
ATSDR	Agency for Toxic Substances and Diseases Registry
AUBPs	AU rich element binding proteins
BaP	Benzo[a]pyrene
bHLH	Basic-helix-loop-helix
βNF	β-naphthoflavone
BSO	buthionine-[*S,R*]-sulfoximine
C12	AhR-deficient Hepa 1c1c7 C12 cells
Cd^{2+}	Cadmium
CHX	Cycloheximide
Cr^{6+}	Chromium
CYP450	Cyptochrome P450
Cyp1a1	Cytochrome P450 1a1
DCF	2′,7′-dichlorofluorescein
DCF-DA	2′,7′-dichlorofluorescein diacetate
DCPIP	2,6-Dichlorophenolindophenol
DMA(V)	Diemthylarsinic acid
DME	Drug metabolizing enzymes

DMSO	Dimethyl sulfoxide
DRE	Dioxin responsive element
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
EH	Epoxide hydrolasae
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
EROD	7-Ethoxyresorufin *O*-deethylase
Gapdh	Glyceraldehydes-3-phosphate dehydrogenase
γ-GCS	γ-glutamylcysteine synthetase
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
Gst a	Glutathione transferase a
H_2O_2	Hydrogen peroxide
HAHs	Halogenated aromatic hydrocarbons
Hepa 1c1c7	Murine hepatoma Hepa 1c1c7
HO	Heme oxygenase
HO-1	Heme oxygenase 1
HSP90	90 kDa heat-shock protein
INAA	Instrumental neutron activation analysis
Keap1	Kelch-like ECH associating protein 1
MAPKs	Mitogen-activated protein kinases
MG-132	Carbobenzoxy-l-leucyl-l-leucyl-leucinal
MM	Multiple myeloma
MMA(III)	Monomethylarsonous acid
MMA(V)	Monomethylarsonic acid
MROD	7-Methoxyresorufin *O*-demethylation
MRL	Minimum risk level
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC	N-acetylcysteine
NF-κB	Nuclear Factor-Kappa B
Nqo1	NAD(P)H:quinone oxidoreductase 1
NLS	Nuclear localization signal
NRE	Negative regulatory elements
Nrf2	Nuclear factor erythroid 2-related factor-2
p23	23-kDa Heat shock protein
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polycyclic aromatic hydrocarbons
PAS	Per-Arnt-Sim
PBS	Phosphate buffered saline
pGudLuc1.1	XRE-luciferase reporter plasmid
PI3K	Phosphotidylinositol 3-kinase
PKC	Protein kinase C
PGs	Prostaglandins
PML	Promyelocytic leukemia
RAR	Retinoic acid receptor
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SSC	saline with sodium citrate
TBS	Tris-buffered saline
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TNB	5-Thio-2-nitrobenzoic acid
WT	Wild-type murine hepatoma Hepa 1c1c7 cells
XAP2	Hepatitis B virus X-associated protein 2
XRE	Xenobiotic responsive element

1.0.0.0. Chapter 1.0

INTRODUCTION

1.0.0.0. INTRODUCTION

1.1.0.0. ENVIRONMENTAL CONTAMINANTS AND THE ARYL HYDROCARBON RECEPTOR (AhR)

Halogenated aromatic hydrocarbons (HAHs) such as: polychlorinated-dibenzo-p dioxins (PCDDs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs); and polycyclic aromatic hydrocarbons (PAHs), are a diverse group of widespread environmental contaminants. Their ubiquitous distribution, fat solubility, and bioaccumulation brand HAHs and PAHs as major health hazards. Exposure to these environmental contaminants produces a range of biological, and often toxic, effects (DeVito et al., 1994, Hankinson, 1995, Safe, 1990). Although often tissue- and species-specific, biochemical evidence consistently attribute these toxic effects to the aryl hydrocarbon receptor (AhR), a cytosolic receptor to which these contaminants bind (Heath-Pagliuso et al., 1998).

1.2.0.0. AhR HISTORICAL PERSPECTIVE

Evidence for the presence of a receptor protein responsible for the activity of HAHs and PAHs was first suggested with the identification of a distinctive biochemical action of HAHs and PAHs, the induction of microsomal hydroxylase activity. Examination of this hydroxylase activity identified genetic polymorphism in mice in which the C57BL/6 strain was more responsive to induction of hydroxylase activity than the DBA/2 inbred mouse strain (Nebert and Gielen, 1972, Nebert et al., 1972, Landers and Bunce, 1991). The 'responsive' phenotype was later deduced to be controlled by a single autosomal gene (Landers and Bunce, 1991).

In 1973, Poland and Glover hypothesized that an induction receptor was the mediator responsible for the reported changes in enzyme activities and toxicities induced by hydrocarbons (Poland and Glover, 1973). After this receptor protein was identified in the C57BL/6 mouse hepatic cytosol (Poland et al., 1976), a hypothesis emerged that would link its binding to hydrocarbons to the observed biochemical and biological changes. It was thus proposed that binding of hydrocarbons to this receptor in the cytosol would induce a conformation change that would allow the receptor to translocate into the nucleus, bind to DNA, and induce transcription (Landers and Bunce, 1991).

Identification of the AhR triggered an explosion of research to identify the biological role of this receptor protein. It was first suggested that the AhR may have evolved to detoxify xenobtiotics. Another speculation was that the AhR serves as a receptor for yet to be identified endogenous ligands and that HAHs and PAHs are merely exogenous ligands that disrupt the physiological function of the AhR (Landers and Bunce, 1991). Identification of important physiological functions of the AhR have verified the latter hypothesis. It is now confirmed that the AhR plays a critical role in liver development (Harstad et al., 2006), female reproduction (Baba et al., 2005), and circadian rhythm (Mukai and Tischkau, 2007).

1.2.1.0. AhR signaling pathway

A conglomerate of studies have identified the AhR as a ligand-activated basic helix–loop–helix (bHLH) protein that exists as cytoplasmic aggregates bound to two 90-kDa heat-shock proteins (HSP90), the 23-kDa heat shock protein (p23), and hepatitis B virus X-associated protein 2 (XAP2). Upon ligand binding, the ligand-receptor complex dissociates from HSP90 and translocates to the nucleus where it associates with the aryl hydrocarbon receptor nuclear translocator (ARNT). The whole complex then acts as a transcription factor that binds to DNA promoter sequences termed xenobiotic responsive elements (XREs), thereby enhancing the transcription of an array of genes (Nebert and Duffy, 1997). Among these genes are those encoding a number of drug metabolizing enzymes (DMEs), including four phase I enzymes: cytochrome P450 1a1 (Cyp1a1), Cyp1a2, Cyp1b1, and Cyp2s1; and four phase II enzymes: NAD(P)H: quinone oxidoreductase (Nqo1), glutathione transferase a (Gst a), cytosolic aldehyde dehydrogenase-3 and UDP-glucuronosyltransferase 1a6 (Figure 1.1) (Nebert and Duffy, 1997, Rivera et al., 2002).

Multiple regions within the AhR protein which aid in protein and DNA binding and hence transcriptional activation have been identified. The bHLH motif, which functions

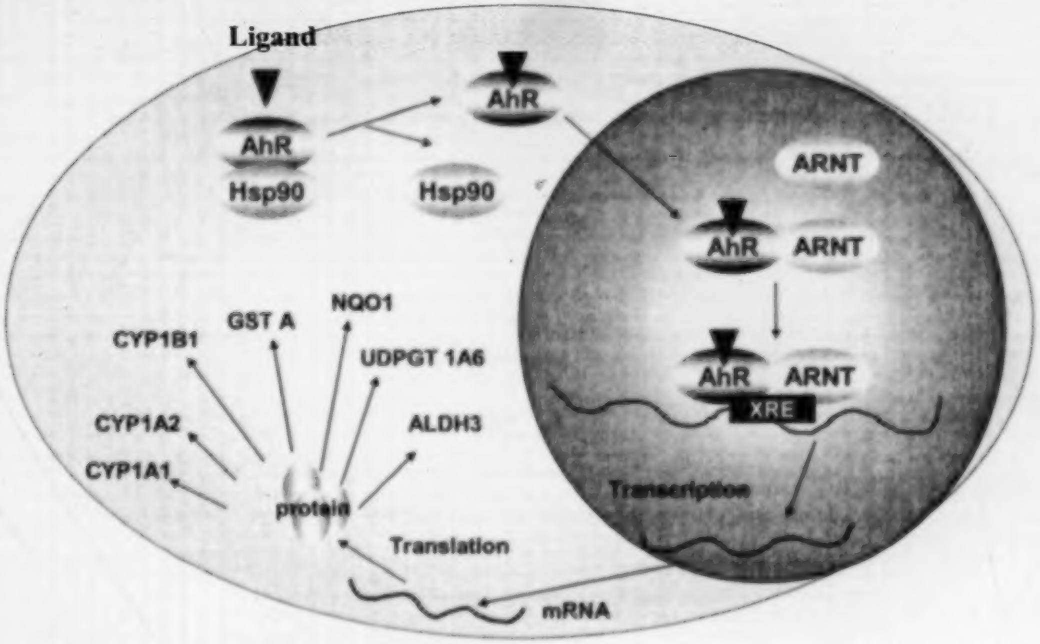
Ligand
AhR
Hsp90
AhR
Hsp90
ARNT
AhR
ARNT
AhR
ARNT
XRE
NQO1
GST A
CYP1B1
UDPGT 1A6
CYP1A2
ALDH3
CYP1A1
protein
Transcription
Translation
mRNA

Figure 1.1. The AhR signaling pathway.

in protein dimerization and DNA binding, is found near the amino terminal of the AhR protein (Figure 1.2) (Fukunaga et al., 1995). Another region, the Per-Arnt-Sim (PAS) domain, is involved in both protein dimerization and ligand binding. In the AhR protein, the PAS domain contains two copies of a 50-amino acid degenerate direct repeat, termed PAS A and PAS B. On the other hand, transcriptional activation is carried out by a glutamine (Q) rich region in the carboxy terminal (Fukunaga et al., 1995).

Within the cytosolic complex, the Hsp90 complex interacts with two distinct domains of the AhR: the PAS-B domain and the bHLH (Kazlauskas et al., 2001). It has been shown that Hsp90 is required to maintain the high affinity ligand binding conformation of the AhR and repress intrinsic DNA binding activity (Kazlauskas et al., 2001, Pongratz et al., 1992) while p23 stabilizes the AhR-Hsp90 interaction (Kazlauskas et al., 1999). XAP2 on the other hand protects the AhR against proteasome-mediated degradation by inhibiting receptor ubiquitination and promotes cytoplasmic retention of the AhR (Kazlauskas et al., 2000).

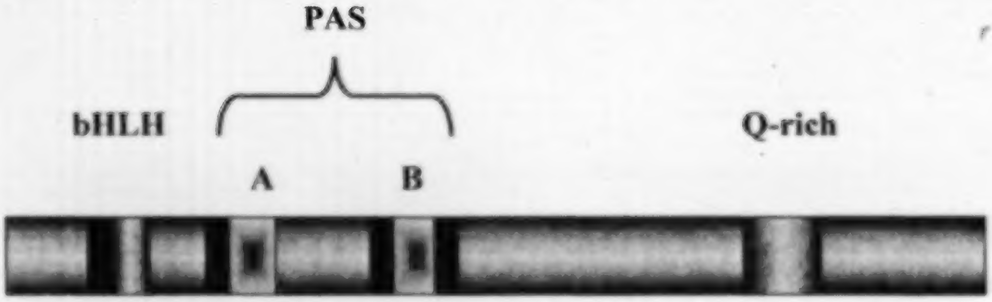

Figure 1.2. Function domains of the AhR protein. Adapted from Fukunaga et al., 1995.

1.2.2.0. Receptor activation and DNA binding

Activation of the AhR is a multi-step process that involves ligand binding, release of the AhR protein from the cytoplasmic complex, and its translocation into the cytoplasm. The ligand binding domain of the AhR exists within the PAS B domain (Dolwick et al., 1993). Ligand binding induces a conformational change that leads to exposure of the nuclear localization signal (NLS) that allows for the active transport of the AhR across the nuclear membrane (Ikuta et al., 1998). Heterodimerization with ARNT leads to DNA binding and subsequent activation of transcription. The recognition sequence of the AhR-ARNT dimmer, the XRE, is also known as the dioxin- or Ah-responsive element (DRE or AhRE). The XRE is asymmetrical (5`-TNGCGTG-3`), suggesting that both the AhR and ARNT bind to different parts of the consensus sequence. It is believed that ARNT binds to the sequence at 5`-GTG-3` while the AhR binds 5` to these nucleotides (Hankinson, 1995). Binding of the heterodimer leads to chromatin remodeling through the recruitment of multiple coactivators, including p300 and p/CIP, and subsequent increase in gene transcription (Hankinson, 2005).

1.3.0.0. CLASSICAL AhR LIGANDS

Various compounds have demonstrated an ability to activate the AhR. Early structure activity relationship analyses using the 'classical' AhR ligands, HAHs and PAHs, suggested that the AhR ligand binding site accommodates planar, aromatic, and hydrophobic compounds within a maximal space of 14 Å x 12 Å x 5 Å. Furthermore, it was revealed that binding affinity was dependent on the electronic and thermodynamic properties of the ligand (Kafafi et al., 1993, Waller and McKinney, 1995). Interestingly, HAHs are more potent inducers of the AhR than PAHs, possibly due to their relative resistance to metabolic inactivation (Denison and Nagy, 2003).

1.3.1.0. Sources of exposure

HAHs and PAHs are the most extensively studied AhR ligands due to their ubiquitous nature. HAHs and PAHs are released into the environment through various natural and synthetic processes during incomplete combustion (ATSDR 2006, 1995). Volcanoes and forest fires are the main natural sources of exposure but represent a minor source of environmental exposure. Synthetic processes serve as the major sources of environmental contamination. HAHs and PAHs are found in tobacco smoke, automobile and truck emissions, and municipal waste water. Wood burning in homes and many industrial processes, such as metal smelting, waste incineration, and paper bleaching are major sources of environmental contamination (ATSDR 2006, 1995).

1.3.2.0. Toxicity

The toxicity of HAHs and PAHs in experimental animals and exposed populations is well established (Table 1.1.). Numerous studies using strains of mice, which differ in their AhR inducibility, implicate the AhR in mediating many of the hydrocarbon-induced toxic effects (Safe, 1990). C57BL/6 mice, containing a high affinity AhR, were more prone than another strain containing a low affinity AhR to PAH-mediated genotoxicity, carcinogenesis, teratogenesis, and other toxicities of the liver and ovaries (Nebert, 1989). Also, AhR-knockout mice were resistant to TCDD-mediated toxicity (Fernandez-Salguero et al., 1996), BaP-induced skin carcinomas (Shimizu et al., 2000), and benzene hemotoxicity (Yoon et al., 2002). PAH-mediated genotoxicity and tumor initiation has been attributed to the induction of xenobiotic metabolizing enzymes by the AhR (Nebert et al., 2000). Independent of enzyme induction, the AhR is associated directly with the formation of oxidative stress and tumor promotion (Poland et al., 1982, Senft et al., 2002).

Table 1.1. Reported toxicities of AhR ligands.

Toxic effect	**Tissue specific effect**	**References**
Death		(Robinson et al., 1975, Thyssen et al., 1981)
Immunotoxicity	Thymic involution Immune suppression	(Holsapple et al., 1991, Kamath et al., 1997, Kerkvliet, 2002)
Genotoxicity		(Maier et al., 2000)
Carcinogenicity	Respiratory tract tumours	(Beems, 1984, Kim and Lee, 1996)
	Hemotological malignancies	(Bertazzi et al., 2001, Thyssen et al., 1981)
	Soft tissue sarcoma	(Fingerhut et al., 1991)
Endocrine disruption	Diabetes mellitus	(Bertazzi et al., 2001)
	Anti-estrogen	(Moran et al., 2003)
	Thyroid hyperplasia	(Nishimura et al., 2003)
Reproductive toxicity	Embryotoxicity Reduced fertility	(Wassenberg and Di Giulio, 2004)
Teratogenicity	Hydronephrosis, cleft palate, small kidneys	(Peters et al., 1999)
Hepatotoxicity	Increased liver weight	(Robinson et al., 1975)
	Hepatocellular necrosis	(Ohbayashi et al., 2007)
Wasting syndrome		(Olsen et al., 1994)
Dermal effects	Chloracne Hyperkeratosis	(Smith et al., 2007, Yamamoto and Tokura, 2003)
Neuropathy	Peripheral and central neuropathy	(Michalek et al., 2001)

1.4.0.0. NON-CLASSICAL AhR LIGANDS

Growing interest in the AhR provoked the search for novel AhR ligands. Surprisingly, the AhR has been found to bind to synthetic compounds whose structure and physiochemical properties differ from the classical ligands, the HAHs and PAHs (Denison and Nagy, 2003). The structural diversity of these 'nonclassical' AhR ligands is clearly evident by their molecular structures. Carbaryl, a carbamate insecticide, primaquine, and omeprazole, are examples of novel AhR ligands whose structure deviates significantly from the classic planar, hydrophobic structure of the HAHs and PAHs (Backlund and Ingelman-Sundberg, 2004, Denison et al., 1998, Werlinder et al., 2001). Not surprisingly however, the majority of these ligands are relatively weak inducers of the AhR. Nonetheless, the identification of these novel ligands paved the way for the identification of endogenous AhR ligands that may induce the AhR during physiological and/or pathological conditions (Denison and Nagy, 2003).

1.5.0.0. ENDOGENOUS AhR LIGANDS

Identification of an active AhR in cell culture and tissue slices in the absence of an exogenous AhR ligand prompted the search for possible endogenous AhR ligands (Singh et al., 1996). A large number of endogenous compounds with the ability to activate the AhR *in vitro* have been identified. Not surprisingly, these structurally distinct classes of compounds are relatively weak inducers of the AhR. Furthermore, their ability to activate the AhR *in vivo* is yet to be confirmed.

Endogenous AhR ligands have been grouped into several categories, including indoles, tetrapyroles, and arachidonic acid (AA) metabolites (Denison and Nagy, 2003). Indole-containing substances are primarily endogenous metabolites of tryptophan. Tryptophan and several of its naturally occurring metabolites, including tryptamine, indole acetic acid, indigo, and indirubin, have been reported to activate the AhR in yeast and mammalian cell cultures (Adachi et al., 2001, Heath-Pagliuso et al., 1998). Products of the heme degradation pathway have been found to activate the AhR signalling pathway. Heme metabolites with the tetrapyrrole structure, including bilirubin and biliverdin, activate the AhR in cultured cells at physiologically relevant concentrations (Phelan et al., 1998, Sinal and Bend, 1997). Hydrophobic products of AA metabolism, most notably lipoxin A4 and prostaglandins (PGs), also activate the AhR (Schaldach et al., 1999, Seidel et al., 2001). In fact, several prostaglandins, including PGG2, PGD3, and PGH1, induced DNA binding of the AhR complex *in vitro* (Seidel et al., 2001). The physiological and/or pathological relevance of the ability of various endogenous metabolites to induce the AhR is currently under intensive investigation. However, a role for the AhR in normal development and physiological/biochemical processes is apparent due to the development of several physiological and developmental abnormalities in AhR knockout animals (Lahvis et al., 2000, Schmidt et al., 1996).

1.6.0.0. AhR-REGULATED GENES

The toxicological relevance of AhR ligands stems from their ability to induce a host of genes, in hepatic and extra-hepatic tissues, through the AhR. Several HAHs and PAHs not only serve as agonists of the AhR, but are also substrates for the induced phase I enzymes. The conversion of AhR ligands into diol epoxide compounds by Cyp1a1, Cyp1a2 or Cyp1b1 results in the formation of covalent adducts when these genotoxic metabolites react with guanines in critical genes, potentially initiating tumorigenesis and other toxic responses (Spink et al., 2002). On the other hand, the induction of phase II enzymes is counterproductive to this process as Nqo1 and Gst a serve as a detoxification mechanism for many mutagenic and carcinogenic metabolites (Spink et al., 2002).

1.6.1.0. Cytochrome P450

Cyp1a1, Cyp1a2, and Cyp1b1 are part of the Cyp450 superfamily of enzymes which comprise 70-80% of phase I DMEs (Nebert and Dalton, 2006). The Cyp450 family obtained its name due to a distinct absorption peak formed at 450 nm in ultraviolet/visible spectra when Cyp450 enzymes are reduced and bound with CO (Garfinkel, 1958, Klingenberg, 1958). Members of the Cyp450 family are named based on their relationships in amino acid sequence. In general, members in a gene family contain more than 40% sequence homology while members of the same subfamily have at least 55%

sequence homology and lie within the same cluster on a chromosome (Nebert et al., 1987).

The Cyp450 are the terminal oxidase of an electron transfer system in the endoplasmic reticulum. They are heme-containing proteins with a single protoporphyrin IX prosthetic group. They are made up of 400-500 amino acids with molecular weights ranging between 45 000 and 50 000 daltons. The heme group is non-covalently bound to a cysteine residue, in a 15 amino acid conserved sequence in apoprotein, and acts as the active site for oxidative metabolism for a wide range of substrates (Gibson and Skett 2001). The metabolic role of the Cyp450 can be summed up as that of the function of a monoxygenase. The reaction can be summarized as:

$$RH+O_2+NADPH\ H^+ \rightarrow ROH+H_2O+NADP^+$$

where RH is an oxidisable substrate and ROH is the oxidized metabolite (Gunsalus and Sligar, 1978). In addition to the above reaction, Cyp450 also catalyzes N-, O-, and S-dealkylation reactions through a two-step reaction, involving the hydroxylation of the alkyl group (Gibson and Skett 2001).

The Cyp1a subfamily comprises of only two members, Cyp1a1 and Cyp1a2. Cyp1a1 is primarily an extrahepatic enzyme since its constitutive expression is low. On the other hand, Cyp1a2 is primarily a hepatic enzyme that is constitutively expressed. Nonetheless, the Cyp1a1 may be induced 8-16 fold in the liver to a level that exceeds the level of both constitutive and inducible expression of hepatic Cyp1a2. In contrast to other members in the Cyp1 family, Cyp1b1 is inducible in numerous tissues, mainly the liver, lung, kidneys, and ovaries (Bhattacharyya et al., 1995). A difference between the three enzymes also arises in their substrate specificity. While Cyp1a2 preferably metabolizes hydrophilic amines, Cyp1a1 and Cyp1b1 metabolize the more hydrophobic PAHs and HAHs (Nebert and Dalton, 2006). Despite difference in their tissue expression, the regulation of the three members in the Cyp1 family is mainly transcriptional, regulated by the AhR.

1.6.1.1. Cyp1a1 regulation

As mentioned before, the induction of Cyp1a1 is regulated through the AhR receptor. Dissection of the mammalian *CYP1A1* gene however reveals multiple regulatory elements which may modulate its expression. Several XRE sequences are located approximately 1 kb upstream of the transcription start site in all mammalian *Cyp1a1* genes (Hines et al., 1988). Interestingly, there are three glucocorticoid responsive elements (GREs) in the human, rat, and mouse *Cyp1a1* gene, which explains why the inducibility of Cyp1a1 by PAHs is modulated by glucocorticoids (Monostory et al., 2005). A negative regulatory element (NRE), located between -560 and -831, inhibits constitutive expression of the Cyp1a1 (Hines et al., 1988). This suppression has been attributed to the nuclear transcription factor Oct-1 which interacts with the NRE (Bhat et al., 1996). Despite thorough evidence on the regulatory mechanisms of Cyp1a1, the regulation of Cyp1a1 expression is not completely understood. Changes in physiological conditions, in the absence of an exogenous ligand, have been shown to induce Cyp1a1. Changes in physiological conditions, including stressful conditions, such as hyperoxia (Hazinski et al., 1995), and suspension of cells (Sadek and Allen-Hoffmann, 1994a, Sadek and Allen-Hoffmann, 1994b) or induction of differentiation (Crawford et al., 1997) increase Cyp1a1 expression in the absence of an exogenous ligand. Whether the induction of Cyp1a1 in these conditions is attributed to the productions of endogenous AhR ligands, or to other regulatory mechanisms is still under investigation.

1.6.1.2. The role of Cyp1 enzymes in HAH- and PAH-mediated toxicity

The Cyp1 enzymes are not only regulated by PAHs, but are also responsible for their activation into toxic metabolites. The role of Cyp1 enzymes in mediating PAH toxicity can be demonstrated from epidemiological studies showing a correlation between the high CYP1 inducibility phenotype in cigarette smokers and cancers of the lung, larynx, and oral cavity (Senft et al., 2002). Xenobiotics have been shown to be activated by the Cyp1 family into genotoxic and carcinogenic metabolites. Carcinogenic PAHs are activated by several drug metabolizing enzymes to highly mutagenic metabolites responsible for their toxicity (Shimada and Fujii-Kuriyama, 2004, Shimada, 2006). The major activation pathway for PAHs, however, is through the formation of diol epoxides by members of the Cyp450 and epoxide hydrolase enzymes (Shimada, 2006). BaP, the prototype carcinogenic PAH, is metabolized by Cyp1a1 to BaP-7,8-oxide which is subsequently hydrolyzed by epoxide hydrolase (EH) to (+)- and (-)-BaP-7,8-diol. Cyp1a1 is then again responsible for the conversion of these metabolic intermediates into the toxic bay region metabolites, (-)-BaP-7,8-diol-9,10-oxide-1, (+)-BaP-7,8-diol-9,10-oxide-2, (+)-BaP-7,8-diol-9,10-oxide-1, and (-)-BaP-7,8-diol-9,10-oxide-2 (Shimada, 2006) (Figure 1.3). All four epoxides are highly mutagenic, yet (+)-BaP-7,8-diol-9,10-oxide-2 was identified as the most tumorgenic of the metabolites (Kapitulnik et al., 1978, Levin et al., 1976). The toxicity of other bay region epoxide metabolites has similarly been reported for other PAHs, including benz[a]anthracene-3,4-diol, benz[b]fluoranthene-9,10-diol, and benzo[c]phenanthrene-3,4-diol (Conney, 1982, Levin et al., 1976, Pelkonen and Nebert, 1982, Shimada, 2006).

Other members of the Cyp1 family are also involved in xenobiotic activation. Cyp1b1 metabolizes PAHs, aryl- and heterocyclic amines to genotoxic metabolites (Shimada et al., 1996). In fact, Cyp1b1-null mice were not able to metabolize 7,12-DMBA and were resistant to the formation lymphomas and other malignancies caused by 7,12-DMBA (Buters et al., 1999). Furthermore, CYP1B1 is the principal precipitant of PAH-mediated immunotoxicity (Fujii-Kuriyama and Mimura, 2005). Interestingly, *in vitro* work demonstrated a 10-fold higher activity for CYP1B1 than CYP1A1 in converting BaP to BaP-7,8-diol in a reconstituted human enzyme system (Shimada et al., 1999). However, similar rates of activation were reported for CYP1A1 and CYP1B1 in the activation of various PAH diols, including (-)-BaP-7,8-diols, 7,12-DMBA-3,4-diol, and B[a]A-3,4-diol (Amin et al., 1995, Conney, 1982). On the other hand, CYP1A2 demonstrated different enzyme kinetics as it had slower rates in the activation of these diols (Shimada, 2006).

6.2.0.0. Nqo1

PAHs and their reactive metabolites, such as those produced by the Cyp1 family, are detoxified by Ugt, Gst, Nqo1, and other phase II enzymes to more polar, readily excretable metabolites. The carcinogenic metabolites BaP 1,3-, 1,6-, and 3,6-quinones are detoxified by Nqo1 (Shimada, 2006). In fact, Nqo1 prevents the formation of BaP quinone-DNA adducts generated by Cyp1a1 (Joseph and Jaiswal, 1994), also evidenced by the fact that Nqo1 deficiency increases BaP-induced carcinogenesis (Long et al., 2000).

Nqo1 is a cytosolic homodimeric flavoprotein involved in cellular defense mechanisms through three distinct mechanisms. It maintains various antioxidants in their reduced, and hence, active states and is involved in the regulation of the tumor suppressor, p53 (Nioi and Hayes, 2004). Also, as its name suggests, Nqo1 also metabolizes the single step, two electron reduction of quinones, thus bypassing the formation of the reactive semiquinone metabolism intermediate (Dinkova-Kostova and Talalay, 2000, Talalay and Dinkova-Kostova, 2004).

1.6.2.1. Quinone metabolism and toxicity

Although abundant in the environment, quinones are also formed in the body through the metabolism of endogenous and exogenous compounds by the Cyp450 (Nioi and Hayes, 2004). For example, 1,6-, 3,6-, and 6,12-quinones are end products of BaP metabolism through a series of reactions involving the Cyp450 (Shimada, 2006). The toxicity of quinones arises from their ability to undergo single electron reduction by various enzymes, including Cyp1a1, Cyp1a2, and Cyp450 reductase, resulting in the formation of reactive semiquinones (Monks and Jones, 2002, Nioi and Hayes, 2004) (Figure 1.4). These metabolite intermediates may directly bind to cellular macromolecules resulting in the formation of DNA and protein adducts and subsequent cellular toxicity. In addition, semiquinones may reduce other molecules resulting in the production of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$) and the hydroxyl radical ($OH^{\cdot}$). Iron, for example, is reduced by semiquionones directly, or indirectly, through

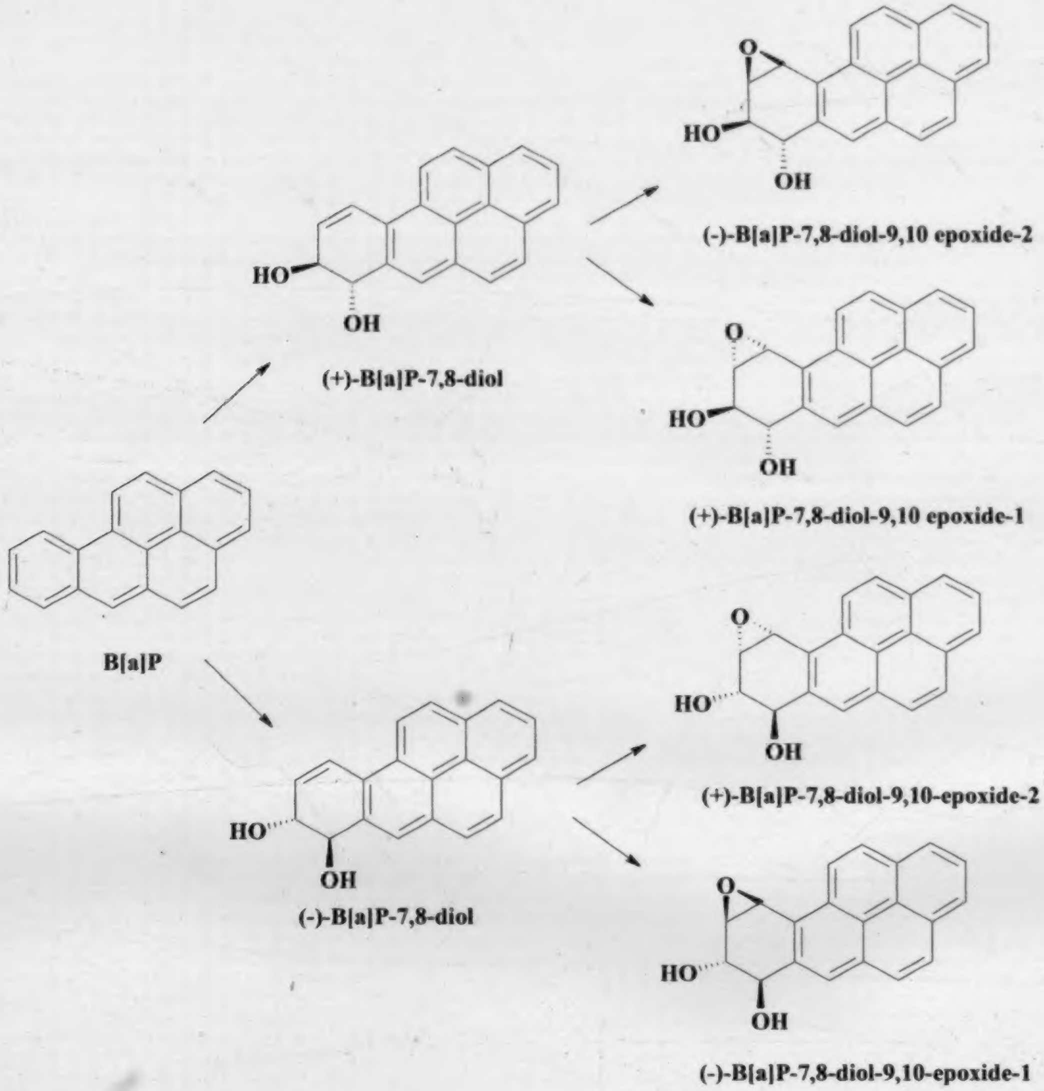
HO
OH
(-)-B[a]P-7,8-diol-9,10 epoxide-2
HO
OH
(+)-B[a]P-7,8-diol
O
HO
OH
(+)-B[a]P-7,8-diol-9,10 epoxide-1
B[a]P
O
HO
OH
(+)-B[a]P-7,8-diol-9,10-epoxide-2
HO
OH
(-)-B[a]P-7,8-diol
O
HO
OH
(-)-B[a]P-7,8-diol-9,10-epoxide-1

Figure 1.3. Metabolism of BaP by EH and Cyp1. Adapted from Baird et al., 2005.

molecular oxygen, which initiates a Fenton reaction leading to the formation of oxygen radicals (Figure 1.4) (Nioi and Hayes, 2004). The oxygen radicals will also in turn attack cellular macromolecules causing enzyme inactivation, lipid peroxidation, and DNA damage. Cells impart protection from the deleterious effects of quinones through the constitutive expression and induction of Nqo1 which, through a single step two electron reduction reaction, catalyzes the conversion of quinones to hydroquinones which are easily conjugated and excreted by the body (Figure 1.4) (Ernster, 1967). Not surprisingly, Nqo1 lessens BaP and BaP-quinone-induced mutagenicity and hence reduces BaP-mediated carcinogenesis (Joseph and Jaiswal, 1994). Also, Nqo1-null mice are more likely to develop BaP-induced skin and internal organ tumors than their wild-type counterparts (Jaiswal, 2004).

1.6.2.2. Nqo1 regulation

Considering its important role in detoxification, it is not surprising that Nqo1 is ubiquitously distributed in all tissues (Jaiswal, 2004). Furthermore, Nqo1 expression can be further induced in response to xenobiotics, antioxidants, prooxidants, and ionizing radiation (Jaiswal, 2004). In fact, Nqo1 is part of a cellular defence mechanism responsible for the induction of dozens of genes in response to stressful stimuli (Jaiswal, 2004). An enhancer region in the human, rat, and mouse *Nqo1* gene, designated as the antioxidant response element (ARE), is responsible for directing Nqo1 basal expression and gene induction by xenobiotics, antioxidants, and stressful stimuli (Jaiswal, 2004). Sequence analysis of the human Nqo1 ARE gene has identified the presence of an embedded perfect activator protein 1 (AP-1) binding site which is missing in the rodent genes (Favreau and Pickett, 1991, Jaiswal, 1991).

The expression of ARE-directed genes is influenced by both the level and the subcellular localization of nuclear factor erythroid 2-related factor 2 (Nrf2) protein (Nioi and Hayes, 2004), although various complexes of nuclear transcription factors, including c-Jun, Jun-B, Jun-D, c-Fos, Fra1, Nrf1, Nrf2, AhR, and the estrogen receptor (ER) bind to the ARE of various genes (Jaiswal, 2000). Nrf2 is a member of the Cap'n'Collar family of basic

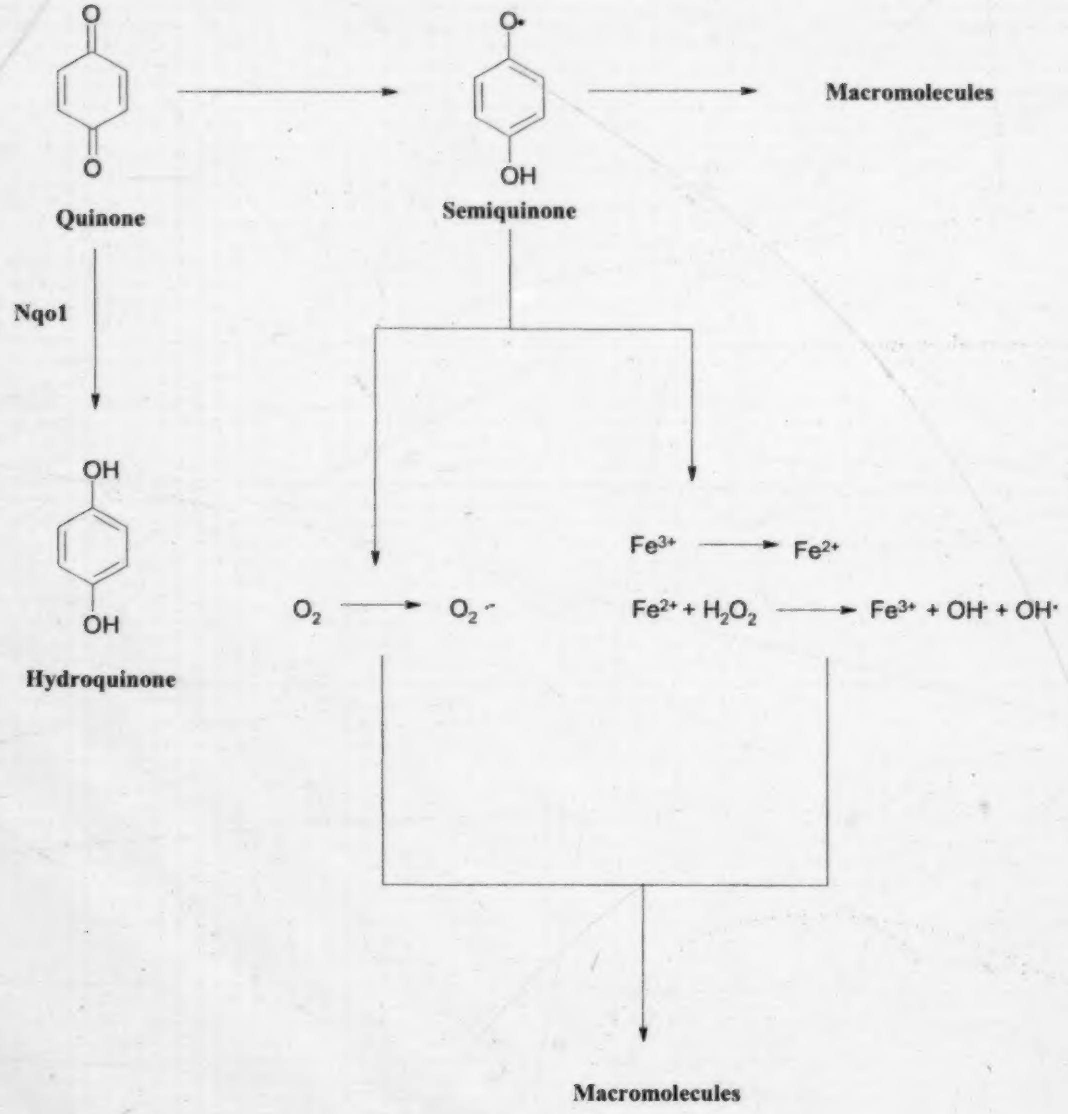
Quinone
Semiquinone
Macromolecules
Nqo1
Hydroquinone
O2 → O2·−
Fe3+ → Fe2+
Fe2+ + H2O2 → Fe3+ + OH− + OH·
Macromolecules

Figure 1.4. Mechanism of toxicity of quinones, and their detoxification by Nqo1. Adapted from Nioi and Hayes, 2004.

region-leucine zipper (bZIP) transcription factors whose activity is tightly controlled by the negative regulator Kelch-like ECH associating protein 1 (Keap1) (Jaiswal, 2000, Nioi and Hayes, 2004). Keap1 is a cytoplasmic factor that interacts with the actin cytoskeleton and Nrf2, thus retaining Nrf2 in the cytoplasm. In addition, Keap1 targets Nrf2 to proteasomal degradation through the ubiquitin pathway (McMahon et al., 2003). Thus, liberation of Nrf2 appears to be a key step in activating ARE-driven gene expression. The exact mechanism by which Nrf2 is released from Keap1 is yet to be determined; however, it has been postulated that during times of oxidative stress, cysteine residues in Keap1 are modified, leading to the dissociation of Nrf2 and its subsequent translocation into the nucleus (Wakabayashi et al., 2004). In addition, certain signaling pathways may contribute to this process. Protein kinase C (PKC)-mediated phosphorylation of Nrf2 has been identified as an important step in the nuclear translocation of Nrf2 during oxidative stress (Huang et al., 2002). Furthermore, activation of mitogen activated protein kinase (MAPK) pathways activate ARE reporter genes by a Nrf2-dependent mechanism (Yu et al., 2000) while phosphotidylinositol 3-kinase (PI3K) is involved in the rearrangement of actin microfilaments and the subsequent nuclear translocation of Nrf2 (Kang et al., 2002).

1.6.2.3. Regulation of Nqo1 through the XRE

The ARE is an important regulator of Nqo1 expression, but some aromatic hydrocarbons, such as TCDD, induce Nqo1 through an AhR- and ARNT-dependent manner (Ma et al., 2004). Not surprisingly, putative XRE sequences have been found in human (Jaiswal, 1991), rat (Favreau and Pickett, 1991), and mouse (Nioi et al., 2003) *Nqo1* promoter gene. Interestingly, XRE-dependent mouse *Nqo1* gene activation is dependent not only on the AhR and Arnt, but also on the presence of Nrf2 as Nqo1 mRNA was not induced in Nrf2-/- mice exposed to 3-methylcholanthrene (Noda et al., 2003). Although the exact mechanism by which a cross-talk between Nrf2 and AhR occurs is not clear, it is likely due to a physical interaction between the ARE and XRE through linker proteins (Nioi and Hayes, 2004).

1.6.3.0. Gst a

Gsts are another group of detoxifying drug metabolizing enzymes with the ability to conjugate electrophiles including those represented by a carbon, sulfur, oxygen, or nitrogen atom. As such, the Gsts' substrate group is extremely large, the majority of which are either xenobiotics and their metabolites or products of oxidative stress. Arene oxides, quinones, epoxides, and hydroperoxides are some of the most toxicologically relevant functional groups susceptible to GSH conjugation (Hayes and Pulford, 1995). The conjugation reaction mediated by Gsts results in the formation of a thioether bond and hence a metabolite that is less reactive and more soluble, so it is easily excreted from the body. GSH conjugation also tags the molecule for export from the cell through multidrug resistance-associated protein (MRP1 and MRP2) (Hayes and Pulford, 1995, Hayes et al., 2005).

Gsts comprise a subset of three superfamilies of enzymes: cytosolic, mitochondrial, and microsomal. Mammalian cytosolic isoenzymes are dimers, categorized in 7 families designated as Alpha (A1-A5), Mu (M1-M5), Pi (P1), Sigma (S1), Theta (T1,T2), Omega (O1-O2) , and Zeta (Z1) (Hayes et al., 2005, Mannervik et al., 1985). In humans, a total of 16 Gst subunits have been identified that form 16 homodimers. In addition, members of the Alpha and Mu classes form a large number of heterodimers with members of the same class (Hayes and Pulford, 1995, Hayes et al., 2005).

1.6.3.1. Regulation of Gst a expression

Gst a1 and a2 are constitutively expressed and are induced by many xenobiotics and physiological stress. Fingerprinting of the promoter region has identified several cis acting elements in the 5'-flanking region of the rat *Gst a2* gene, including the ARE, the XRE, the glucocorticoid responsive element (GRE), and a Barbie box element responsive to phenobarbital (Hayes and Pulford, 1995). The promoter of the murine *Gst a1* gene, on the other hand, contains two tandomly arranged ARE and a Barbie box element. Surprisingly, although the intron and exon structures of the human and rodent *GSTA* genes are similar, there are striking differences in their mode of transcriptional regulation.

Human *GST A* genes do not contain an ARE or XRE (Suzuki et al., 1994). Rather, transcriptional activation of the human *GSTA* genes occurs through an AP-1 and AP-2 consensus sequence and a GRE (Whalen and Boyer, 1998). Moreover, hepatic nuclear factor 1 has been located in the human *GST A* promoter region and functions as an enhancer (Clairmont et al., 1994).

1.7.0.0. HEAVY METALS

Although there are numerous studies examining the toxic effects of individual AhR ligand forms, there are relatively few reports of the combined toxic effects of AhR ligands and other environmental co-contaminants. Among these, environmental co-contaminants of most concern are heavy metals, typified by arsenite (As^{3+}), cadmium (Cd^{2+}), and chromium (Cr^{6+}). Heavy metals are found in air, water, soil, and food. Both As^{3+} and Cd^{2+} are among the top seven most hazardous environmental contaminants listed on the Agency for Toxic Substances and Disease Registry (ATSDR) (Klaassen 2001)

1.7.1.0. As^{3+}

Arsenic is widely distributed in the environment and can be found as organic and inorganic compounds in the trivalent (arsenite) or the pentavalent (arsenate) states (Klaassen 2001). Inorganic arsenite is released into the environment from primary copper, zinc, and lead smelters and glass, pesticide, and herbicide manufacturing (Klaassen 2001). It also found in cigarette smoke, arsenic-treated wood, and agricultural fertilizers (Patrick, 2003).

Exposure to arsenic species occurs through the oral and inhalation routes. Depending on the chemical form, 50-95% of ingested arsenite or arsenate is absorbed through the oral route, although absorption of less soluble species, such as lead arsenate, tends to be much lower. Dermal absorption on the other hand is quite low, reaching only 4.5% after 24 h. Following absorption, arsenic is distributed evenly to all tissues, with little tendency to accumulate in organs (EPA 2001), although it has been shown to concentrate in nails and hair (Klaassen 2001).

Inorganic arsenite is transported intracellularly via aquaporins 7 and 9 (Liu et al., 2004). Intracellulary, As^{5+} is reduced to As^{3+}, which is subsequently methylated by an oxidative reaction to monomethylarsonic acid (MMA(V)). Subsequent reduction/oxidation and methylation reactions convert MMA(V) to monomethylarsonous acid (MMA(III)) and dimethylarsinic acis (DMA(V)) (Spiegelstein et al., 2003). Methylated arsenite may then flow out of the cell down its concentration gradient through aquaporin 9 (Liu et al., 2004), or may form glutathione conjugates which are pumped out of the cell by ATP-dependent transporters (Liu et al., 2001). Urinary excretion of inorganic and methlated arsenite accounts for 75% of the absorbed arsenite dose. A small percentage of the absorbed dose is excreted in the feces (EPA 2001), in sweat, and by desquamation of skin (Klaassen 2001).

The toxicity of trivalent inorganic arsenicals has been known for thousands of years. Ingestion of 70-180 mg of inorganic arsenite results in an acute illness characterized by the presence of fever, anorexia, hepatomegaly, cardiac arrythmias and cardiovascular failure, peripheral neuropathy, various hematopoietic effects, and possibly death (Franzblau and Lilis, 1989, Klaassen 2001). Chronic arsenite exposure has also been linked to the development of hematopoietic abnormalities, neurotoxicity, cirrhosis and hepatoportal sclerosis, endocrine disruption, cardiovascular disease, and immunotoxicity (Chen and Wang, 1990, Klaassen 2001).

Not surprisingly, exposure to arsenite induces malignant transformation in multiple organ systems. Arsenite-induced hyperpigmentation and hyperkeratosis may lead to the formation of Bowen's disease and basal cell and squamous cell carcinomas (Yu et al., 2006). Arsenite exposure has also been linked to hepatocellulary carcinoma, angiosarcoma, bladder cancer, and epidermoid bronchogenic carcinoma (Chen and Wang, 1990, Klaassen 2001, Marsh et al., 1998).

1.7.1.1. As^{3+} toxicity and oxidative stress

The exact cellular mechanisms of arsenite's toxicity are not completely understood. It is known that arsenite induces deletion mutations, chromosomal aberrations, and DNA methylation, and alters signal transduction, cellular differentiation, and gene expression (Klaassen 2001, National Academy of Sciences 2001). All these mechanisms, however, are associated with the development of oxidative stress (Ercal et al., 2001).

As^{3+} has been shown to stimulate the production of superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2) in various cellular systems including human vascular smooth muscle cells (Lynn et al., 2000), human keratinocyte cells (Pi et al., 2003), human–hamster hybrid cells (Liu et al., 2001), and HEL30 cells (Corsini et al., 1999). Human studies confirm these findings with a reported increase in serum lipid peroxide levels in populations exposed to high arsenite concentrations in drinking water (Pi et al., 2002) and a decrease in plasma antioxidant capacity (Hsueh et al., 1998).

As^{3+} induces the production of ROS as a result of its intrinsic ability to accumulate in the mitochondria and alter cellular respiration (National Academy of Sciences 2001). As^{3+} binds thiol groups of the dihydrolipoic acid cofactor of pyruvate dehydrogenase, inhibits succinic dehydrogenase in the electron transport chain, and competes with phosphate during oxidative phosphorylation (Klaassen 2001, Patrick, 2003). The resulted inhibition of mitochondrial respiration stimulates ATPase, decreases ATP production, and results in the production of ROS (Klaassen 2001, Patrick, 2003).

The role of ROS in mediating the cellular mechanisms of As^{3+} toxicity has been extensively studied at the molecular and supramolecular levels. 8-Hydroxy-2'-deoxyguanosine, a major product of ROS-induced DNA base-modification, was significantly higher in arsenic-related skin neoplasms than in arsenic-unrelated Bowen's disease (Matsui et al., 1999). As^{3+}-induced oxidative stress is responsible for the apparent changes in cellular signaling pathways and aberrations in the cell cycle (Jung et al., 2003, Navarro et al., 2006). Moreover, superoxide dismutase, catalase, glutathione, and other antioxidants, decrease O_2^{-} production and preclude the development of chromosomal damage in fibroblasts (National Academy of Sciences 2001). As^{3+} is also known to alter the function of various transcription factors, affect promoter function of various genes, and modify enzyme activities through oxidative mechanisms (Mei et al., 2002, Pi et al., 2003, Schuliga et al., 2002).

1.7.2.0. Cd^{2+}

In addition to As^{3+}, Cd^{2+} is one of the most toxic environmental contaminants (Jarup et al., 1998). Environmental exposure to Cd^{2+} is mainly a consequence of occupational exposure to Cd^{2+}-contaminated fumes in the lead and zinc refining industries and during the manufacture of paint pigments, cadmium-nickel batteries, and electroplating (Klaassen 2001). Cd^{2+} is also found in cigarette smoke, providing another source of exposure. Unfortunately, the greatest human exposure remains food. Soil contamination from fallouts, Cd^{2+}-contaminated irrigation water, Cd^{2+}-containing fertilizers, and sewage sludge results in the bioaccumulation of the heavy metal in vegetables, fruits, and grains (Klaassen 2001, Patrick, 2003). Shellfish, animal meat and organs (specifically the liver and kidneys) also contain high concentration of Cd^{2+} (Klaassen 2001). Worldwide populations have a dietary intake of up to 100 μg of Cd^{2+} in non-polluted areas and up to 200 μg in polluted areas, levels which exceed the recommended minimum risk level (MRL) of 0.2 μg/kg/day, provided by the ATSDR (ATSDR 1999, Jarup et al., 1998).

Unlike As^{3+}, Cd^{2+} displays poor oral bioavailability, with only 5-6% of the ingested dose absorbed, although absorption may be higher in individuals with dietary deficiencies in iron and calcium. On the other hand, 5-20% of Cd^{2+} inhaled in aerosols and as much as 50% of Cd^{2+} in cigarette smoke is absorbed (EPA 2001, Klaassen 2001). Absorbed Cd^{2+} is distributed throughout the body, particularly to the liver and kidneys, by binding to red blood cells and high molecular weight proteins, especially albumin (EPA 2001, Klaassen 2001). Folllowing distribution to the liver, Cd^{2+} induces the formation of metallothionein which consequently complexes with Cd^{2+}. The Cd^{2+}-metallothioenein complex is then stored in the liver or is transported to the kidneys where it accumulates (EPA 2001, Klaassen 2001). Persistent accumulation of Cd^{2+} results in a high body burden and a half-life of up to 30 years (EPA 2001, Klaassen 2001). As expected, excretion is slow and occurs by both the urinary and fecal routes.

Due to its ubiquitous distribution and prolonged half-life, Cd^{2+} exposure is associated with multi-organ toxicities. Acute Cd^{2+} toxicity from occupational exposure or incidental ingestion of Cd^{2+}-containing drinks has been reported (Klaassen 2001, Patrick, 2003, Wittman and Hu, 2002). Upper gastrointestinal distress, dyspnea, chemical pneumonitis, pulmonary edema, dysuria, polyuria, dizziness, and headache have been reported (Patrick, 2003, Wittman and Hu, 2002). Unlike its acute effects, chronic toxicities of Cd^{2+} are of major health concern. Cd^{2+} circulates in the blood bound to erythrocytes or albumin and is deposited in the liver. In the liver, Cd^{2+} induces and binds to metallothionein, resulting in accumulation of the metal, up to 3000 fold (Klaassen et al., 1999). Over time, Cd^{2+} - metallothionein complexes are released into the blood and transported to the kidneys where they accumulate in the lysosomes (Klaassen 2001). Cd^{2+} also accumulates in the bone, pancreas, adrenals, and placenta culminating in its long half life of 10-30 years and multi-organ toxicities (Jarup et al., 1998).

Cd^{2+} causes glomerular dysfunction and tubular cell degeneration and fibrosis (Jarup et al., 1998, Klaassen 2001). In the skeletal system, Cd^{2+} has been linked to the development of osteomalacia and osteoporosis (Jarup et al., 1998, Klaassen 2001). Its skeletal effects are reminiscent of its ability to affect vitamin D metabolism, increases urinary excretion of calcium, and interfere with calcium incorporation into bone cells (Nogawa et al., 1987). Chronic Cd^{2+} exposure is also a risk factor for the development of chronic obstructive pulmonary disease, hypertension and cardiovascular disease (Klaassen 2001, Nishijo et al., 1995, Thun et al., 1989). Not surprisingly, epidemiological studies have shown a link between respiratory exposure to Cd^{2+} and lung cancer (Thun et al., 1985). In animals, Cd^{2+} induces the formation of injection site sarcomas and tumors of the prostate, testes, and kidneys (Coogan et al., 1995, Waalkes et al., 1999a, Waalkes et al., 1999b).

1.7.2.1. Cd^{2+} toxicity and oxidative stress

Similar to As^{3+}, the mechanisms of Cd^{2+} toxicity are not completely defined, although multiple cellular mechanisms may be involved. Again, these cellular mechanisms are not exclusive as cellular oxidative stress seems to be the intermediate modulator of all cellular changes observed, including chromosomal damage (Fowler, 1978). Cd^{2+} induces oxidative stress in cultured human cells (Bagchi et al., 2000) and increases lipid peroxidation in the liver, kidney, brain, lung, heart, and testes of animals (Ercal et al., 2001, Shaikh et al., 1999). Cd^{2+}-induced oxidative stress is a consequence of inhibition of antioxidant enzymes and alterations in thiol status. Decreased activity of glutathione peroxidase has been observed in Cd^{2+}-exposed populations (Patrick, 2003). Cd^{2+} also inhibits other antioxidant enzymes, including catalase and superoxide dismutase (Casalino et al., 2002). It is also proposed that Cd^{2+} may replace iron and copper in cellular proteins, causing an increase in free copper and iron ions which may induce the production of ROS through the Fenton reactions (Casalino et al., 1997). A recent study has shown a direct correlation between Cd^{2+}-induced DNA nicking and the production of $OH^{\cdot}$. Free radical generation leads to enhanced expression of inflammatory chemokines and cytokines, the oxidation of nucleic acids, damage in DNA repair mechanisms, and eventual genotoxic effects (Fowler, 1978).

1.7.3.0. Cr^{6+}

Chromium is naturally abundant in the earth's crust, mainly in the form of Cr^{3+} (Dayan and Paine, 2001) while various industrial processes using chromite ore release chromium into the air in the form of Cr^{6+}. Chromite ore is converted into sodium chromate and dichromate for use as an anticorrosive agent in cooking instruments; and for the production of chromium alloys, chrome pigment, chrome salts for tanning leather, and wood preservatives (Klaassen 2001). As with Cd^{2+}, the major source of Cr^{6+} human exposure is food, as it is estimated that humans ingest less than 100 µg of Cr^{6+} daily (Klaassen 2001). In areas of low Cr^{6+} exposure, average blood Cr^{6+} levels are 20-30 µg/L, a level that is significantly increased in heavily polluted areas (Klaassen 2001).

Cr^{3+} and Cr^{6+}, which possess different chemical and biological properties, are both of significance to human health. Cr^{3+} is an essential mineral required for the maintenance of normal carbohydrate metabolism (Dayan and Paine, 2001) as it is thought to enhance insulin-stimulated insulin receptor tyrosine kinase activity (Davis and Vincent, 1997). As such, The Food and Nutrition Board of the National Research Council (NRC) has recommended a safe and adequate intake of chromium of 50-200 µg/day (National Research Council 1989).

Absorption of chromium compounds varies depending on the chemical species, ranging from 0.5-2% for dietary Cr^{3+} compounds, to 2-10% for chromates (CrO_4^{2-}). In general the oral absorption of Cr^{6+} compounds is much higher than Cr^{3+} compounds. Chromates enter the cells through anion channels while Cr^{3+} compounds are absorbed by passive diffusion and phagocytosis. Absorption also occurs through the lungs and dermal routes but is gernally very poor. Once absorbed, chromium compounds are distributed to all body organs and is eventually excreted in the urine with a half-life of 30-40 h (ATSDR 1994).

Although minimal amounts of Cr^{3+} are absorbed orally, an oral dose of 50-70 mg/kg body weight of chromates is considered lethal in humans. Death usually occurs due to severe diarrhea and hemorrhage into the gastrointestinal tract, caused by the corrosive nature of Cr^{6+}, which induce cardiovascular shock (Dayan and Paine, 2001, Sharma et al., 1978). Toxic oral doses of Cr^{6+} also induce liver and kidney necrosis (World Health Organization 1990). Allergic contact dermatitis occurs with exposure to Cr^{6+} and is independent of the dose (Siegenthaler et al., 1983). Allergic reactions also occur in the respiratory tract, elicited in the form of asthma, rhinitis, bronchospasm, and pneumonia. Chronic exposure to Cr^{6+} is also associated with the formation of tumors of the respiratory tract (Dayan and Paine, 2001, World Health Organization 1990).

1.7.3.1. Cr^{6+} toxicity and oxidative stress

All known toxicities of chromium are attributed to the +6 form. Cr^{6+} enters cells more readily than Cr^{3+}. At physiological pH, Cr^{6+} exists as a chromate ion, with an overall -2 charge, resembling sulfate and phosphate ions and as such is transported across the cell membranes via the anionic transporters (Alexander and Aaseth, 1995). Cr^{6+} is ultimately reduced to Cr^{3+} by multiple mechanisms that involve glutathione and cysteine, ascorbic acid (AscA), Cyp450, hemoglobin, and glutathione reductase (International Agency for Research on Cancer 1990). Eventually, all Cr^{6+} is converted to Cr^{3+}, but the nature and concentration of the reducing agent will determine whether Cr^{6+} is converted directly to Cr^{3+} or whether it is converted to various intermediates, such as Cr^{5+} and Cr^{4+}, before being converted to Cr^{3+} (Zhitkovich, 2005). Interestingly, Cr^{6+}-induced DNA strand breaks, DNA-DNA and DNA-protein cross-links do not occur in cell-free systems in the absence of reducing agents (Dayan and Paine, 2001). Although Cr^{3+} interacts directly with phosphate groups and nitrogen bases in DNA (Tamino et al., 1981), the formation of ROS during the reduction process is thought to mediate the observed genotoxicity (Leonard et al., 2000). $O_2^{\cdot-}$ and H_2O_2 are produced when Cr^{6+} is reduced to Cr^{5+}. It is also suggested that Cr reduction intermediates may undergo Haber-Weiss and Fenton-type reactions, resulting in the production of $O_2^{\cdot-}$, $OH^{\cdot}$, and OH^{-}. Hypothetical reactions that involve the different valence forms of Cr include (Krepkiy et al., 2003, Leonard et al., 2000):

$$Cr^{6+} + GSH \rightarrow GS^{\cdot} + Cr^{5+}$$

$$GS^{\cdot} + GSH \rightarrow GSSG^{\cdot} + H^{+}$$

$$GSSG^{\cdot} + O_2 \rightarrow O_2^{\cdot-} + GSSG$$

$$2\,O_2^{\cdot-} + 2H^{+} \rightarrow H_2O_2 + O_2$$

$$Cr^{3/4/5/6+} + O_2^{-} \rightarrow Cr^{3/4/5+} + O_2$$

$$Cr^{3/4/5+} + H_2O_2 \rightarrow Cr^{4/5/6+} + OH^{\cdot} + OH^{-}$$

1.7.4.0. An oxymoron, the therapeutic benefits of heavy metals

Despite its toxicity, As^{3+} has been used as a therapeutic agent for more than 2400 years in Traditional Chinese Medicine and in ancient Greek and Roman Times (Shen et al., 1997). Unexpectedly, As^{3+}, as arsenic trioxide, is being used for the treatment of acute promyelocytic leukemia (APL) and multiple myeloma (MM) (Hussein, 2001, Murgo, 2001, Shen et al., 1997).

Evidence supporting the use of As^{3+} in APL is overwhelming. A daily dose of 10 mg (0.15 mg/kg) of arsenic trioxide was shown to produce a 72.7% clinical remission rate in newly diagnosed patients treated for 6-weeks, for up to 2 cycles (Niu et al., 1999). Furthermore, 85% of relapsed parients achieved complete remission (Niu et al., 1999, Soignet et al., 2001). The mechanism(s) by which As^{3+} exhibits its anti-cancer effects are still at large. One APL-specific mechanism involves degradation of the promyelocytic leukemia (PML)-retinoic acid receptor α (RARα) fusion protein, a product of t(15:17) translocation, causing the induction of differentiation and apoptosis (Chen et al., 1997).

The anticancer effects of As^{3+} are not exclusive to APL. As^{3+} is also effective in the treatment of myelodysplastic syndromes (Vey et al., 2006) and primary liver and gallbladder cancers (Qian et al., 2001). Studies have also documented its effectiveness in inhibiting proliferation and inducing apoptosis of human colonic, breast, and pancreatic cancer cells (Li et al., 2002, Li et al., 2004). The mechanism of action of As^{3+} in cancers lacking the PML-RARα fusion protein may be multiple and various. Various reports have linked the generation of ROS to the efficacy of As^{3+} in cancer (Jing et al., 1999). The apoptotic effects of As^{3+} are related to its effect on mitochondrial function and the production of ROS and consequent release of cytochrome c and activation of caspase-9 and caspase-3 (Gazitt and Akay, 2005, Jing et al., 1999),

Paradoxically, however, AscA enhances the anticancer effect of As^{3+} (Hussein, 2001). AscA enhanced the anti-lymphoma and antimyeloma effects of As^{3+} without additional toxicity to normal tissue (Bachleitner-Hofmann et al., 2002, Campbell et al., 2007). The anticancer effect of As^{3+} undeniably occurs by multiple mechanisms, and the mechanism by which AscA enhances these effects remains another question to be answered. These therapeutic benefits, however, highlight a gap in our knowledge on the pharmacology of heavy metals in the human body.

1.8.0.0. ENVIRONMENTAL CO-EXPOSURE TO HEAVY METALS AND AhR LIGANDS

Environmental co-contamination with metals and AhR-ligands has been reported worldwide. Small airborne particulates of a small industrialized North Italian town, and in areas of the Czech Republic where coal is used for heating and power, were found to contain traces of PAHs and heavy metals (Monarca et al., 1997, Sram et al., 1996). PAHs and heavy metals were also found in sediment samples from sites close to Göteborg, Sweden (Magnusson et al., 1996), in the river Meuse in western Europe (Schilderman et al., 1999), the Dniester River in the former Soviet republic of Moldova (Sapozhnikova et al., 2005), the New Bedford Harbor in Massachusetts (Ho et al., 1997), and in the Sheboygan River system in east-central Wisconsin (Schrank et al., 1997). The tissues of wild city pigeons exposed to air pollution in the Netherlands were found to contain both PAHs and heavy metals (Schilderman et al., 1997). An alarming study from Catalonia, Spain reported that exposure of children to PAHs and heavy metals through seafood was higher than that for other age groups, based on total intake per unit of body weight (Marti-Cid et al., 2007).

With the growing human exposure to AhR ligands and heavy metals, emerging evidence suggests that AhR ligand and metal co-exposure generates different biological responses than is expected based on the toxicological mechanisms of each class evaluated separately. Modulation of AhR ligand-dependent toxicities by heavy metals may occur by multiple mechanisms. It has been shown that As^{3+} increases benzo[a]pyrene (BaP)-induced DNA adducts, likely by multiple mechanisms (Maier et al., 2002). One of these mechanisms includes the inhibition of DNA repair mechanisms. Shen et al. demonstrated the ability of MMA(III) to inhibit (the reactive metabolite of BaP) BaP-diol epoxide-DNA adduct repair by inhibiting p53 phosphorylation (Shen et al., 2007). Interestingly, As^{3+} increased the cellular uptake of BaP-diol epoxide, implying that increased cellular uptake may be one mechanism by which As^{3+} increases BaP mutagenicity (Shen et al., 2006). However, the role of heavy metals on the metabolism of AhR-ligands is yet to be examined. Any influence of metals on the capacity of AhR ligands to induce AhR-regulated genes, and hence alter their metabolism, will influence the carcinogenicity and mutagenicity of the AhR ligands. Thus, an important determinant of the role of heavy metals in modulating the toxicity of AhR ligands is their effect on the enzymes responsible for the metabolism of AhR ligands.

1.8.1.0. Modulation of drug metabolizing enzyme expression by heavy metals

1.8.1.1. Transcriptional mechanisms

Many studies have demonstrated the ability of heavy metals to alter Cyp450 function. Nonetheless, the mechanisms by which such modulations occur remain unclear. As^{3+}

inhibited the induction of CYP1A1 mRNA and activity and hindered the activation of the CYP1A1 promoter by benzo[k]fluoranthene in HepG2 cells (Bessette et al., 2005). In Hepa-1 cells, TCDD-mediated induction of Cyp1a1 was not affected by neither As^{3+} nor Cd^{2+}, even at high concentrations, although As^{3+} inhibited TCDD-mediated induction of luciferase activity in cells stably transformed with an XRE luciferase reporter construct (Maier et al., 2000). Interestingly, work by the same group demonstrated the ability of As^{3+} to induce AhR nuclear translocation and binding to the *Cyp1a1* gene promoter, resulting in a weak induction in Cyp1a1 mRNA in Hepa-1 cells (Kann et al., 2005). High concentrations of As^{3+} have also been shown to induce transcriptional activation of the CYP1A1 promoter and a XRE promoter construct in HepG2 stably transfected cells (Tully et al., 2000). Controversial results were also reported with Cd^{2+}. In Hepa-1 cells, Cd^{2+} had no discernable effect on TCDD-mediated induction of Cyp1a1 mRNA and AhR-dependent luciferase activity (Maier et al., 2000) although it induced CYP1A1 transcription in HepG2 cells (Vincent et al., 1997). Also in HepG2 cells, Cd^{2+} induced transcriptional activation of the CYP1A1 promoter and a XRE promoter construct in stably transfected cells, in a dose-dependent manner (Tully et al., 2000). Alternatively, Cr^{6+} decreased TCDD-induced Cyp1a1 mRNA in Hepa-1 cells by inhibiting gene transcription, although it did not alter the DNA-binding competence of the AHR (Maier et al., 2000). Yet in HepG2 cells, Cr^{6+} induced transcriptional activation of the CYP1A1 promoter and a XRE promoter construct in stably transfected cells (Tully et al., 2000).

Fewer, albeit comparable, studies have examined the effect of heavy metals on the expression of Nqo1 and Gst a. As^{3+} and Cd^{2+} potentiated TCDD-induced Nqo1 mRNA and activated an ARE-driven beta-galactosidase gene reporter system in Hepa-1 cells (Maier et al., 2000). Similarly, both metals also activated GSTA1 promoter in HepG2 cells (Tully et al., 2000). Cr^{6+}, on the other hand, inhibited Nqo1 mRNA in Hepa-1 cells (Maier et al., 2000) and Gst in the liver of Wistar rats (Anand, 2005) but induced the GSTA1 promoter in HepG2 cells (Tully et al., 2000).

1.8.1.2. Post-transcriptional mechanisms

Alteration of gene expression may occur not only at the transcriptional level, but at any stage up to the expression of protein activity. mRNA transcripts are labile units, subject to degradation within the cell. Interestingly, mRNA degradation is a regulated process, since the half-lives of some mRNAs are known to change throughout the cell cycle, and can be changed in response to extracellular signals (Wilusz et al., 2001). Very few studies have examined the effect of heavy metals on the degradation of mRNA. In Hepa-1 cells, Cr^{6+} had no significant effect on half-life of Cyp1a1 and Nqo1 mRNA in TCDD-induced cells (Maier et al., 2000). Although no studies have been reported, it is likely that heavy metals may alter the degradation of mRNA by a multiple of mechanisms. It has been previously shown that heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) binds the 3'untranslated region of Cyp2a5 mRNA, preventing its digestion by RNAse and prolonging the half life of the transcripts (Aida and Negishi, 1991, Geneste et al., 1996, Glisovic et al., 2003). Interestingly, increased binding of hnRNP A1 to mRNA serves an important role in ensuring cell survival during the stress response, such as that seen in arsenite-stressed cells (Guil et al., 2006). Whether hnRNP A1, or other hnRNPs, serve a role in the posttranscriptional regulation of Cyp1a1 remains to be investigated.

1.8.1.3. Post-translational mechanisms

Post-translational effects of the heavy metals on AhR-regulated genes have been documented in various cell lines. In the human breast cancer cell line, T-47D, As^{3+} had no significant effect on the BaP-induced CYP1A1 and CYP1B1 mRNA but inhibited the activity of both enzymes in a dose-dependent manner (Spink et al., 2002). Similarly, in HepG2 cells and cultured human hepatocytes, As^{3+} and Cd^{2+} inhibited CYP1A1 protein and activity by various PAHs without altering its mRNA levels (Vakharia et al., 2001a, Vakharia et al., 2001b). Attempts to identify putative mechanisms responsible for the posttranslational modifications of CYP1A1 by heavy metals have identified heme oxygenase 1 (HO-1) as a possible culprit. Decrease in CYP1A1 activity by heavy metals has been inversely correlated with an increase in HO-1 levels, implicating the catabolism of heme in the down-regulation of Cyp450 activity (Spink et al., 2002).

1.9.0.0. OXIDATIVE STRESS, TOXICITY, AND HOMEOSTATIC MECHANISMS

Heavy metals are not the sole inducers of ROS, but their toxicity derives from their ability to saturate homeostatic mechanisms that control the production of these noxious by-products. ROS are generated in living systems by UV light, X-rays, and gamma rays; by neutrophils and macrophages during inflammatory reactions; by Cyp450 metabolism of endogenous and exogenous compounds; and as by-products of electron transport chain activity in the mitochondria (Cadenas, 1989). ROS maintain beneficial effects by participating in the cellular response to noxia and the regulation of several signalling pathways. High concentrations of ROS, however, mediate damage to nucleic acids, proteins, and lipid membrane (Valko et al., 2006). The antioxidant defence system, consisting of enzymatic and non-enzymatic antioxidants, is part of the cells' homeostatic mechanisms to keep the production of ROS under check. Oxidative stress is a result of an imbalance between free radical production and the antioxidant defence system.

1.9.1.0. The antioxidant defence system, enzyme antioxidants

The most important enzyme antioxidants include superoxide dismutase (SOD), catalase, and glutathione peroxidase. SOD catalyzes the dismutation of O_2^{-} to the less reactive products, H_2O_2 and O_2, with high reaction rates (Tasiopoulos et al., 2000). There are three forms of SOD in humans: cytosolic Cu,Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD. Catalase, which is found in the peroxisome, catalyzes the degradation of H_2O_2 to H_2O and O_2. H_2O_2 is also a substrate for selenium-dependent glutathione peroxidase (GPx), which serve as the main source of protection when oxidative stress is of reduced intensity. GPx reduces H_2O_2 and organic peroxides (ROOH) to water and alcohol, respectively (Valko et al., 2006).

1.9.2.0. The antioxidant defence system, Non-enzymatic antioxidants

Non-enzymatic antioxidants are of equal importance as enzymatic antioxidants in the cellular antioxidant system. In fact, glutathione metabolism is one of the most important effectors in the antioxidant defence system. GSH, or γ-glutamylcysteinyl glycine, is a tripeptide synthesized by γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase in a two-step reaction (Griffith, 1999, Lu, 1999). Mammalian γ-GCS is a heterodimer, consisting of a heavy, catalytically active subunit (γ-GCS_H) and a light subunit (γ-GCS_L) which modulates substrate binding (Griffith, 1999). Interestingly, the 5' flanking region of the human γ-GCS_H contains AREs and is thus responsive to the presence of oxidant agents (Rahman et al., 1996). GSH synthesis is also controlled by substrate availability. L-cysteine availability is substantially lower than L-glutamate and glycine and is thus regarded as a limiting factor for GSH synthesis (Tateishi et al., 1974). Interestingly, because GSH is taken up poorly by cells, replenishing of cellular GSH levels can often be achieved by administering L-cysteine precursors, such as N-acetyl-L-cysteine (NAC).

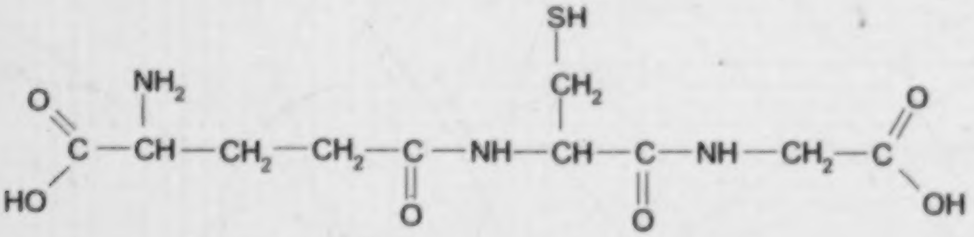

Figure 1.5. Structure of the GSH tripeptide. Adapted from (Lu, 1999).

GSH is abundant in the major soluble antioxidant in the cytoplasm, nuclei, and mitochondria (Barceloux, 1999). GSH plays a major role in the cellular defence against reactive electrophiles by reacting directly with $OH^{\cdot}$ and O_2^1, cooperating with GPx in the reduction of H_2O_2 and lipid peroxides, and serving as the conjugate cofactor in Gst-catalyzed reactions (Griffith, 1999, Lu, 1999). GSH is also involved in reducing Vitamins C and E back to their active forms (Valko et al., 2006). The reaction of GSH with a radical forms thyil radicals that may dimerize to form oxidized glutathione (GSSG):

$$GSH + R^{\cdot} \rightarrow GS^{\cdot} + RH$$

$$GS^{\cdot} + GS^{\cdot} \rightarrow GSSG$$

GSH is regenerated from GSSG by GSSG reductase which maintains glutathione in a predominantley reduced state and as such, redox cycling does not usually alter cellular

GSH levels (Kehrer and Lund, 1994). However, high levels of oxidative stress efficiently diminish GSH levels and cause a rapid increase in GSSG (Griffith, 1999). The ratio of GSH to GSSG is thus a good *in vivo* indicator of oxidative stress (Clemens and Landolph, 2003).

Other significant non-enzymatic antioxidants include vitamins C and E and. Vitamin C, also known as AscA, is a water soluble antioxidant and thus works in the aqueous environment (Kojo, 2004). AscA is a di-acid ($AscH_2$) that exists mainly as $AscH^-$ at physiological pH. $AscH^-$ reacts with free radicals and is converted to the poorly reactive radical $AscH^{\bullet -}$ (Valko et al., 2006). AscA is also involved in the antioxidant defence system through its ability to regenerate Vitamin E. The most active form of Vitamin E, α-tocopherol, is reduced by lipid peroxyl radicals. AscA is thus able to regenerate α-tocopherol to its reduced and active state (Kojo, 2004). Interestingly, some *in vitro* studies have reported a pro-oxidant effect of AscA caused by an increase in free metal ions and the induction of lipid hydroperoxide decomposition (Kang et al., 1998). However, most *in vivo* studies reported a protection against DNA, protein, and lipid oxidative damage even in the presence of redox-active metals (Suh et al., 2003).

1.9.3.0. ROS and gene expression

It is generally accepted that high concentrations of ROS are implicated in cell toxicity and death. At low concentrations, however, ROS play important physiological roles by acting as secondary messengers, regulating the activity of transcription factors and hence the activity of certain gene promoters. Some of the genes altered by ROS include those involved in cellular proliferation and survival (Valko et al., 2006). Serine/threonine kinases, such as the MAPKs are important mediators of ROS-stimulated alterations in gene expression. MAPKs are activated by ROS through receptor and non-receptor tyrosine kinases such as growth factor receptors and Src kinases, respectively, and Ras, a small GTPase (Torres, 2003). Akt (protein kinase B), another serine/threonine kinase, is also activated by ROS, but through PI3K (Valko et al., 2006). PKC represents a family of serine/threonine kinases, activated by lipid cofactors, Ca^{2+}, phosphorylation, and protein-protein interactions (Ron and Kazanietz, 1999). Activation of various signalling pathways by ROS culminates in the regulation of nuclear transcription factors, and hence gene expression. Some of these transcription factors include nuclear transcription factor (NF)-kB, AP-1, p53, and Nrf2 (Valko et al., 2006).

1.9.4.0. ROS and protein function

In addition to their role in modifying gene expression through transcriptional mechanisms, ROS have a direct effect on the expressed proteins. The modification of proteins by ROS leads to changes in protein translocation and turnover and activation or inactivation. H_2O_2 is capable of reacting with all amino acids, including aliphatic, aromatic, and sulphur-containing amino acids. Extraction of an electron from C-H bonds results in the formation of carbon radicals and eventually carbonyl products or protein hydroperoxides (Schoneich and Sharov, 2006). The best characterized modification of protein function by ROS occurs through Cys residues. Oxidation of protein sulfhydryls results in the formation of protein sulfonic acids and thyil radicals (Hamann et al., 2002). One important modification of protein function is the inactivation of protein phosphatases by ROS. Oxidation of Cys residues in phosphatases leads to the formation of intramolecular and intermolecular disulfide bonds and inhibition of protein function (Valko et al., 2006). Peroxynitrite, a product of a reaction between nitric oxide and superoxide, has also been shown to oxidize Cys residues and inactivate vital enzymes including creatine kinase (Konorev et al., 1998), tryptophan hydroxylase (Kuhn et al., 1999) Cyp450 (Daiber et al., 2000), Gst (Wong et al., 2001) and N-acetyltransferases (Dupret et al., 2005).

1.10.0.0. REFERENCES

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1.11.0.0. RATIONALE, HYPOTHESES, AND OBJECTIVES

1.11.1.0. Rationale

HAHs and PAHs are a diverse group of widespread environmental contaminants that produce a range of toxic effects. Most of these toxic effects are mediated by the AhR, a cytosolic receptor to which these contaminants bind. By binding to the AhR, these AhR ligands induce the transcription of Cyp450 enzymes responsible for their metabolism into toxic intermediates. The induction of phase II drug metabolizing enzymes is counterproductive to this process and as such, the toxicity oh AhR ligands is regulated by a coordinated balance between the phase I and phase II drug metabolizing enzymes. Although numerous studies have examined the toxic effects of individual AhR ligands, there are relatively few reports of the combined toxic effects of AhR ligands and other environmental contaminants. Aromatic hydrocarbon and heavy metal co-exposure generates different biological responses than is expected based on the toxicological mechanisms of each class evaluated separately. Any influence of metals on the capacity of AhR ligands to induce AhR-regulated genes will influence the carcinogenicity and mutagenicity of the AhR ligands. Thus, there is a need to understand the mechanisms driving these responses to define the exact role of AhR ligands and metals in carcinogenicity and mutagenicity.

1.11.2.0. Hypotheses

Hypothesis 1: As^{3+}, Cd^{2+} and Cr^{6+} disrupt the coordinated balance of AhR-regulated gene-induction.

Hypothesis 2: Heavy metals are involved in the modulation of AhR-regulated genes at various steps of the AhR signalling pathway.

Hypothesis 3: Heavy metals induce the formation of ROS, which are involved in the modulation of AhR-regulated genes.

1.11.3.0. Specific Objectives

1. To determine the effect of heavy metals on the induction of AhR-regulated genes by various AhR ligands, at the mRNA, protein, and activity levels.
2. To identify molecular mechanisms involved in the modulation of AhR-regulated genes by heavy metals.
3. To examine the role of ROS in the modulation of AhR-regulated genes by heavy metals.

1.11.4.0. Experimental plan

To test the hypotheses, *in vitro* studies were carried out in the murine Hepa 1c1c7 cell line. These cell lines offer the principal model for the investigation of the regulatory mechanisms of AhR-regulated genes due to the availability of mutant cells, Hepa 1c1c7 C12, possessing AhR at 10% of the levels of those found in Hepa 1c1c7 wild-type cells. The expression of Cyp1a1, Nqo1 and Gst a was determined at the mRNA, protein, and activity levels using combinations of various AhR ligands and the heavy metals As^{3+}, Cd^{2+} and Cr^{6+}. To determine the level at which these metals act, studies were carried out at the transcriptional, posttranscriptional, and posttranslational levels using inhibitors of transcription and translation. Finally, the production of ROS and their role in the modulation of AhR-regulated genes was examined, using modulators of oxidative stress.

1.11.5.0. Significance

Establishing the interaction between heavy metals and AhR ligands is the first step to determine the ability of heavy metals to influence the carcinogenicity and mutagenicity of AhR ligands. Identifying the mechanisms involved in the modulation of AhR-regulated genes will aid in the development of preventative strategies and new treatment modalities for AhR-ligand –mediated toxicities.

2.0.0.0. Chapter 2.0

MODULATION OF ARYL HYDROCARBON RECEPTOR-REGULATED GENE EXPRESSION BY ARSENITE, CADMIUM, AND CHROMIUM

A version of this chapter has been published: Elbekai, R.H. and El-Kadi, A.O., 2004. *Toxicology*. 202, pp. 249-269.

2.1.0.0 Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated helix–loop–helix protein that exists as cytoplasmic aggregates bound to two 90-kDa heat-shock proteins (HSP90), the cochaperone p23 and the 43-kDa protein termed hepatitis B virus X-associated protein. Upon agonist binding, the agonist-receptor complex dissociates from HSP90 and translocates to the nucleus where it associates with the aryl hydrocarbon receptor nuclear translocator (ARNT). The whole complex then acts as a transcription factor that binds to DNA promoter sequences termed xenobiotic responsive elements, thereby enhancing the transcription of the responsive genes. Among these genes are those encoding a number of drug metabolizing enzymes, including three phase I enzymes: cytochrome P4501A1 (Cyp1a1), Cyp1a2 and Cyp1b1; and four phase II enzymes: NAD(P)H: quinone oxidoreductase (Nqo1), glutathione *S*-transferase Ya subunit (Gst a), cytosolic aldehyde dehydrogenase and UDP-glucuronosyltransferase 1A6 (Nebert and Duffy, 1997).

Although the induction of phase II enzymes serves as a detoxification mechanism for many mutagens, carcinogens and other toxic compounds, the induction of Cyp1a1, Cyp1a2 and Cyp1b1 is counterproductive to this process. Several AhR ligands are environmental contaminants that not only serve as agonists of the AhR, but, with the exception of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) which is poorly metabolized, are also substrates for the induced phase I enzymes. The conversion of AhR ligands into diol epoxide compounds by Cyp1a1, Cyp1a2 or Cyp1b1 results in the formation of covalent adducts when these genotoxic metabolites react with guanines in critical genes, potentially initiating tumorigenesis and other toxic responses. Such responses include birth defects, immune suppression, and endocrine disruption (Spink et al., 2002).

Although there are numerous studies examining the toxic effects of individual AhR ligand forms, there are relatively few reports of the combined toxic effects of AhR ligands and other environmental co-contaminants. Among these, environmental co-contaminants of most concern are heavy metals, typified by arsenite (As^{3+}), cadmium (Cd^{2+}), and chromium (Cr^{6+}).

In recent studies, metals have been shown to modify CYP450 function. Cd^{2+} induced CYP1A1 transcription in HepG2 cell lines but decreased total hepatic CYP450 levels and testosterone hydroxylase activity in rats (Vincent et al., 1997). CYP1A1 activity was also diminished by Cd^{2+} in rats (Iscan and Coban, 1992), mice (Iscan et al., 1995), and guinea pigs (Iscan et al., 1993). Acute As^{3+} administration has also been shown to decrease hepatic CYP450 (Falkner et al., 1993a). It inhibited the benzo[a]pyrene (BaP)-mediated induction of CYP1A1 and CYP1B1 in T-47D human breast cancer cells (Spink et al., 2002) and of CYP1A1 in HepG2 cells (Vakharia et al., 2001a). It also inhibited the 3-methylcholanthrene (3-MC)-mediated induction of CYP1A in primary cultures of rat hepatocytes (Jacobs et al., 1999). Conversely, As^{3+} potentiated the induction of guinea pig pulmonary CYP1A1 by β-naphthoflavone (βNF) (Falkner et al., 1993a). Similar to the other two metals, Cr^{6+} inhibited TCDD mediated induction of CYP1A1 (Maier et al., 2000).

All three metals have also been shown to alter the expression of phase II drug metabolizing enzymes (Maier et al., 2000). As^{3+} increased Nqo1 activity in the liver and kidneys of rats and total GST activity in rat kidneys (Falkner et al., 1993a). In addition, Cd^{2+} increased the mRNA levels of Gst a in rat alveolar epithelial cells (Shukla et al., 2000).

Emerging evidence suggests that AhR ligand and metal co-exposure generates different biological responses than is expected based on the toxicological mechanisms of each class evaluated separately. Any influence of metals on the capacity of AhR ligands to induce AhR-regulated genes will influence the carcinogenicity and mutagenicity of the AhR ligands. Thus, there is a need to understand the mechanisms driving these responses to define the exact role of AhR ligands and metals in carcinogenicity and mutagenicity.

As a first step to establishing the interaction between heavy metals and AhR ligands, we undertook extensive studies to determine the effect of heavy metals, mainly As^{3+}, Cd^{2+} and Cr^{6+}, on the induction of AhR-regulated genes by the AhR ligands: BaP, 3-MC, βNF, and TCDD, in murine hepatoma Hepa 1c1c7 cells. These cell lines offer the principal model for the investigation of the regulatory mechanisms of AhR-regulated genes due to the availability of mutant cells, Hepa 1c1c7 C12 (C12), deficient in AhR. The availability of these mutant cells allows the study of events in a signaling cascade dependent on the presence of both partners (AhR and ARNT) in the heterodimeric transcription factor.

2.2.0.0. Materials and methods

2.2.1.0. Chemicals

Sodium arsenite, cadmium chloride, chromium trioxide, benzo[a]pyrene, 3-methylcholanthrene, β-nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, bovine serum albumin, cumene hydroperoxide, Dulbecco's modified Eagle's medium base, 7-ethoxyresorufin, fluorescamine, glucose, reduced glutathione, glutathione reductase, 2,6-dichlorophenolindophenol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 7-methoxyresorufin, protease inhibitor cocktail, and anti-goat IgG peroxidase secondary antibody were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). Tris hydrochloride, agarose, and sodium azide were purchased from EM Science (Gibbstown, NJ). Tween-20 was from BDH Inc. (Toronto, ON). Amphotericin B, resorufin, β-naphthoflavone, and 100X vitamin supplements were purchased from ICN Biomedicals Canada (Montreal, Quebec). Gentamicin sulfate, penicillin–streptomycin, L-glutamine, MEM non-essential amino acids solution, fetal bovine serum, TRIzol reagent, and the random primers DNA labeling system were purchased from Invitrogen Co. (Grand Island, NY). Hybond-N nylon membranes and chemiluminescence Western blotting detection reagents were from Amersham Canada (Oakville, ON). [α-^{32}P]dCTP (3000 Ci/mmol) was supplied by Perkin-Elmer (Boston, MA). Bromophenol blue, β-mercaptoethanol, glycine, acrylamide, *N'N'*-bis-methylene-acrylamide, ammonium persulphate, nitrocellulose membrane (0.45 μm), and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA). CYP1A1 goat anti-mouse polyclonal primary antibody (G-18) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Skim milk was obtained from DIFCO Laboratories (Detroite, MI). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

2.2.2.0. Cell culture

Hepa 1c1c7 cell wild-type (WT) and AhR-deficient Hepa 1c1c7 C12 cell lines (C12), generously provided by Dr. O. Hankinson at the University of California (Los Angeles, CA), were maintained in Dulbecco's modified Eagle's medium (DMEM), without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamycin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified incubator. The medium was changed every 2 days and the cells were subcultured every 3 days at a 1:3 ratio.

2.2.3.0. Chemical treatments

Cells were treated in serum free medium alone or with an AhR ligand dissolved in DMSO, metals dissolved in double de-ionized water, or both. The various metals were added 30 min prior to treatment with an AhR ligand, when applicable. The AhR ligands were maintained in DMSO at −20 °C until use, but the metals were prepared freshly for each treatment. In all treatments, the concentration of double de-ionized water in treatment medium did not exceed 0.5% (v/v) while the DMSO concentration did not exceed 0.05% (v/v). For enzyme activity assays, the duration of chemical exposure was 24 h. However, RNA was extracted only 6 h after chemical treatment.

2.2.4.0. Cell cytotoxicity assay

To determine the maximum non-toxic concentration of metals, the effect of metals on cell viability was determined using the MTT assay, as previously described (Vakharia et al., 2001b). The assay measures the conversion of MTT to formazan crystals by enzymes in the mitochondria of metabolically active cells. Briefly, WT and C12 cells were seeded into 96-well microtiter cell culture plates and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. The medium was replaced with serum free medium, and cells were subsequently treated with varying concentrations of As^{3+}, Cd^{2+}, or Cr^{6+}. The cells were further incubated for 24 h and the medium was again removed and replaced with serum free medium containing 1.2 mM of MTT dissolved in PBS (pH 7.2). After 2 h of incubation, the formed crystals were dissolved with isopropanol. The intensity of the colour in each well was measured at a wavelength of 550 nm using the BIO-TEK Instruments EL 312e microplate reader.

2.2.5.0. Determination of EROD and MROD activities

The Cyp1a1-dependent 7-ethoxyreorufin *O*-deethylase (EROD) and 7-methoxyresorufin *O*-demethylation (MROD) activities were performed on intact, living cells as described previously (Sinal and Bend, 1997). Enzymatic activity was normalized for cellular protein content which was determined using a modified fluorescent assay (Lorenzen and Kennedy, 1993).

2.2.6.0. Preparation of cellular homogenates for enzyme activity assays

Cells were grown for 48 h in 6-well plates before treatment with AhR ligands and/or metals. After the elapsed treatment period, cells were washed with PBS and kept in 0.5 ml of homogenization buffer in a −80 °C freezer for at least 24 h. Thawed cells were extracted and homogenized with a Kontes homogenizer and then centrifuged at 10,000 × *g* for 20 min. The supernatant fractions were then removed and protein concentrations in the supernatant were determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as the standard. The extracted cellular homogenates were stored in a −20 °C freezer until they were used for the determination of Nqo1 and Gst a enzyme activities.

2.2.7.0. Determination of glutathione S-transferase a activity

Spectrophotometric assay for Gst a activity using cumene hydroperoxide as a substrate was conducted as previously described (Beaumont et al., 1998). The reaction was performed in 0.1 M potassium phosphate buffer (pH 7.0), containing sodium azide, at 25 °C in a total volume of 1 ml. The rate of enzyme-catalyzed reaction was determined by subtracting the rate of reaction occurring in the absence of glutathione reductase. The reaction mixture contained 0.2 mg of cell homogenate protein with 0.5 mM cumene hydroperoxide, 1 mM reduced glutathione, 0.1 mM NADPH and 0.3 unit of glutathione reductase. The time course of NADPH disappearance was measured at 340 nm for 90 s using an extinction coefficient of 6.2 per mM/cm.

2.2.8.0. Determination of NAD(P)H: quinone oxidoreductase 1 activity

Nqo1 activity was determined by the continuous spectrophotometric assay of Ernster (Ernster, 1967), which quantitates the reduction of 2,6-dichlorophenolindophenol (DCPIP) by 0.01 mg of cell homogenate protein in the presence of β-nicotinamide adenine dinucleotide phosphate (NADPH; 200 μM) and flavin adenine dinucleotide (FAD; 5 μM). The rate of reduction of DCPIP (40 μM) in 1 ml of Tris–HCl buffer (pH 7.8, 25 mM) containing 0.1% (v/v) Tween-20 and 0.023% bovine serum albumin was monitored for 90 s at 600 nm with using an extinction coefficient of 2.1 per mM/cm.

2.2.9.0. RNA extraction and analysis

After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen Co.), and quantified by measuring the absorbance at 260 nm. Northern blot analysis of total RNA was performed as described elsewhere (Sambrook et al., 1989). Briefly, aliquots of 20 μg of RNA were separated in a denaturing (0.22 M formaldehyde) agarose (1.1%) gel and transferred to Hybond-N nylon membranes. The RNA was cross-linked to the membranes using the UV Stratalinker 2400, followed by baking at 65 °C for 2 h. Prehybridization of the membranes was carried out in a solution containing: 6× SSPE (0.9 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.02 M EDTA); 50% de-ionized formamide; 5× Denhardt's reagent (0.1% polyvinylpyrrolidone, 0.1% ficoll, 0.1% bovine serum albumin); 0.5% sodium dodecyl sulfate (SDS); and 100 μg/ml sheared salmon sperm DNA, for at least 4 h at 42 °C. Hybridization with the [^{32}P]-labeled cDNA probes was carried out in the same solution, minus Denhardt's reagent, for 16–24 h at 42 °C. The membranes were then washed twice at room temperature in a solution containing 2× SSPE and 0.5% SDS for 5 min, the first wash, and 15 min for the second wash. This was followed by a 30 min wash in 0.1× SSPE, 0.5% SDS at 42 °C and a final 30 min wash in

0.1× SSPE, 0.5% SDS at a temperature of 62 °C for 30 min. The washed membranes were rinsed in a 0.1× SSPE solution, dried, sealed in plastic wrap and visualized by exposure to Kodak Biomax MS film. Hybridization signals were quantified relative to the signals obtained for glyceraldehydes-3-phosphate dehydrogenase (gapdh) mRNA.

The cDNA probes for mouse Cyp1a1, HO-1, and gapdh mRNA were generously provided by Dr. John R. Bend (University of Western Ontario, London, ON). The cDNA probe for mouse Gst a mRNA was provided by Dr. David Eaton (University of Washington, Seattle, WA). The mouse Nqo1 cDNA probe was prepared from previously described primer sequences (Chen et al., 1994) by reverse transcription coupled to PCR amplification using the primers FNAD(P)H:Nqo1 5'-GCCATGGCGGCAGAAGAGCCCTG-3' (F1; forward) and BNAD(P)H:Nqo1 5'-CTTATTTTCTAGCTTTGATCTG-3' (R1; reverse) containing Nco 1 and Hind III restriction sites, respectively. All probes were [^{32}P]-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions.

2.2.10.0. Western immunoblotting analysis

Cells treated for 24 h with an AhR ligand and/or metal were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µl/ml of protease inhibitor cocktail. The cellular homogenates were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Proteins (25 µg) were resolved by denaturing electrophoresis, as described previously (Sambrook et al., 1989). Briefly, the cytosol supernatants were dissolved in 1X sample buffer, boiled for 5 min, separated by 7.5% SDS-PAGE bis-acrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 24 h at 4 °C in blocking buffer containing 5% skim milk powder, 2% bovine serum albumin and 0.05% (v/v) Tween-20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris–base). After blocking, the blots were incubated with a primary polyclonal goat anti-mouse Cyp1a1 antibody for 2 h at room temperature in TBS containing 0.05% (v/v) Tween-20 and

0.02% sodium azide. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody was carried out in blocking buffer for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (Amersham, Arlington Heights, IL).

2.2.11.0. Statistical analysis

All results are presented as mean ± S.D. The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc comparison. The differences were considered significant when $P<0.05$.

2.3.0.0 Results

2.3.1.0. Effect of metals on cell viability

To determine the optimal concentrations to use in our studies, we evaluated the effects of metals on WT and C12 cell viabilities at 1, 5, and 10 μM concentrations for As^{3+}, and 1, 5, and 25 μM concentrations for Cd^{2+} and Cr^{6+}, during a 24-h incubation period using the MTT assay. In WT cells (Figure 2.1A), all three metals did not affect cell viability at all concentrations tested (>90% viability). However, the metals differentially affected the cell viability of C12 cells (Figure 2.1B). For C12 cells, there appears to be strong dose-response relationships with correlation coefficients of 0.9994, 0.9249, and 0.9955 for As^{3+}, Cd^{2+} and Cr^{6+} treatments, respectively. As^{3+} was the most toxic and Cr^{6+} was the least toxic to C12 cells, as evident by the LC_{50} values indicated in Figure 2.1. All subsequent studies on WT cells were conducted at 1, 5, and 10 μM of As^{3+} and 1, 5, and 25 of μM Cd^{2+} and Cr^{6+}. Studies on C12 cells were conducted at the 5 μM concentration for all metals. The results depicted here indicate that with the concentrations used in this study, the biological effects of the metals are not due to reduced cell viability.

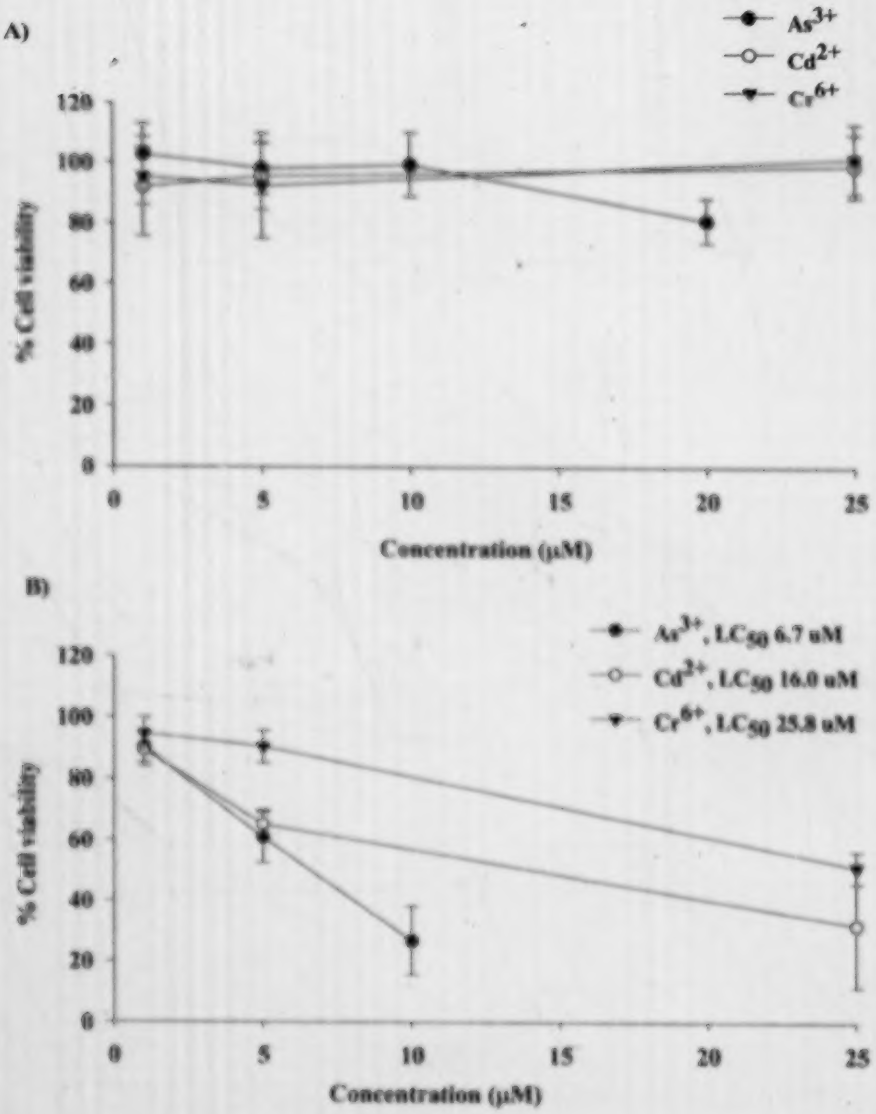

Figure 2.1. Effect of As^{3+}, Cd^{2+}, and Cr^{6+} on murine hepatoma Hepa 1c1c7, WT, (A) and Hepa 1c1c7, C12, (B) cell viability, as a function of metal concentration. Viability was tested 24 h after treatment with metals, using the MTT assay. The concentrations of As^{3+}, Cd^{2+} and Cr^{6+} required to produce a 50% reduction in the viability of C12 cells (LC_{50}) were computed to be 6.7, 16.0, and 25.8 μM, respectively. Data are expressed as percent of untreated control, which is set at 100%, ±S.D. (*n* = 8).

2.3.2.0. Effect of metals on constitutive and inducible Cyp1a1 catalytic activities

Due to the vital role of Cyp1a1 in the production of mutagenic species from AhR ligands, we evaluated the effect of metals on the induction of this enzyme. The effect of metals on AhR ligand-mediated induction of EROD activity is shown in Figure 2.2. In all experiments, EROD activity was undetectable in untreated cells, but all AhR ligands alone effectively induced EROD activity. When co-administered with an AhR ligand, all metals decreased the induction of EROD activity mediated by the AhR ligands in a metal- and AhR ligand-dependent manner. Cd^{2+} was the most effective at inhibiting AhR ligand-mediated induction of EROD activity, followed by Cr^{6+}. As^{3+} was the most effective at abating the induction of EROD activity by BaP while Cd^{2+} and Cr^{6+} were most effective at inhibiting the induction by TCDD and 3-MC, respectively.

The AhR ligands alone were more effective at inducing EROD than MROD activity (Figures 2.2 and 2.3). As^{3+}, Cd^{2+} and Cr^{6+} inhibited MROD induction, although dose-dependency was only observed with Cd^{2+}. MROD induction by all four AhR ligands was completely abolished by 25 μM Cd^{2+} (Figure 2.3). Cr^{6+} inhibited TCDD, 3-MC, βNF, and BaP mediated MROD induction, at the 25 μM concentration, by 44, 61, 53, and 59%, respectively. Hence, Cd^{2+} was more effective at inhibiting MROD induction while Cr^{6+} was more effective at inhibiting EROD induction. Although As^{3+} significantly inhibited AhR ligand-mediated induction of MROD activity at all concentrations tested ($p<0.05$), the 5 μM concentration was more effective than the 10 μM concentration at inhibiting MROD induction. Moreover, As^{3+} at 10 μM significantly induced MROD activity with TCDD and 3-MC, compared to As^{3+} at 5 μM ($p<0.05$).

It is important to note that none of the metals alone caused a significant induction of EROD or MROD activities.

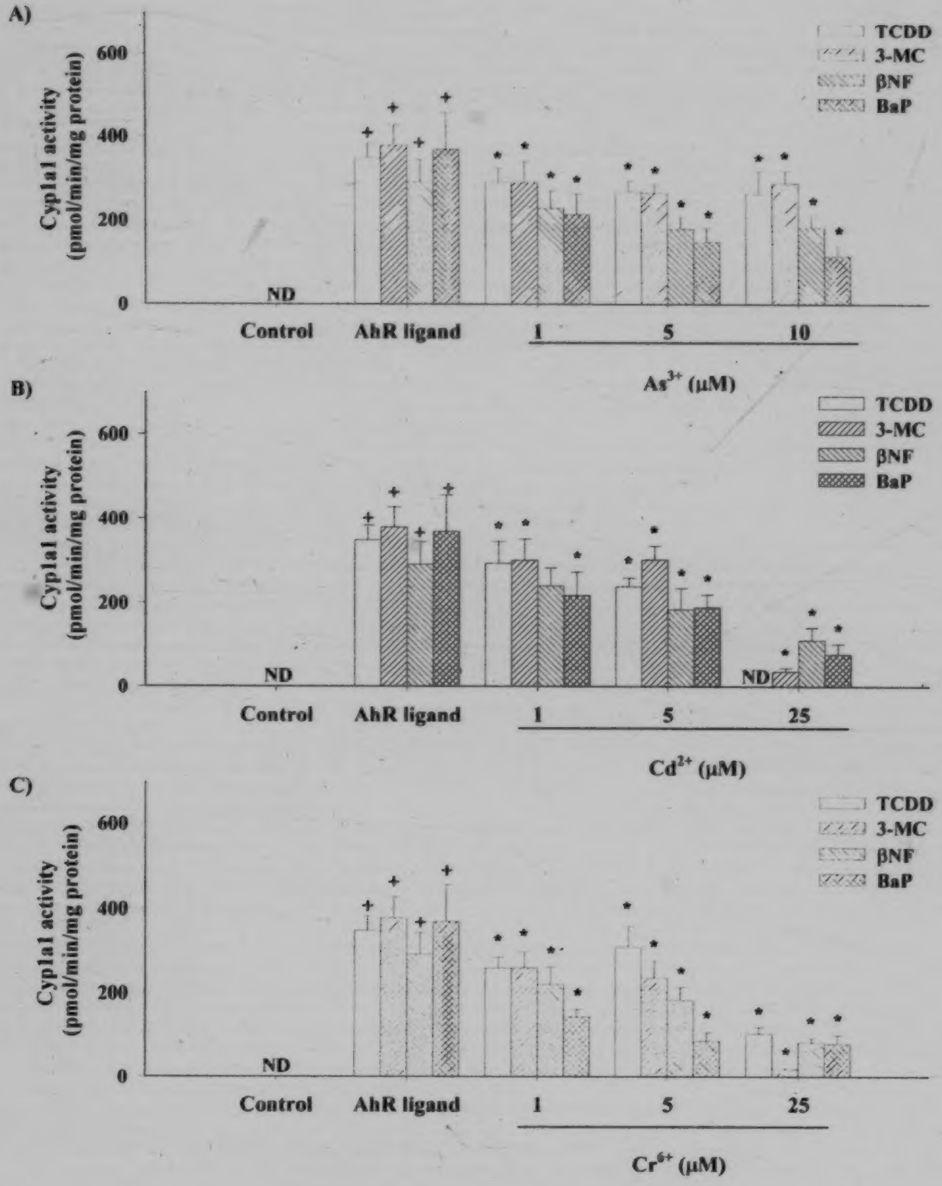

Figure 2.2. Effect of As^{3+} (A), Cd^{2+} (B), and Cr^{6+} (C) on the induction of Cyp1a1 (EROD) activity in murine hepatoma Hepa 1c1c7 by TCDD (0.1 nM), 3-MC (0.25 µM), βNF (10 uM), or BaP (1 µM). Values represent mean activity ± S.D. (*n*= 8). $^{+}p< 0.05$ compared to control; $^{*}p<0.05$ compared to AhR ligand. ND: not detectable.

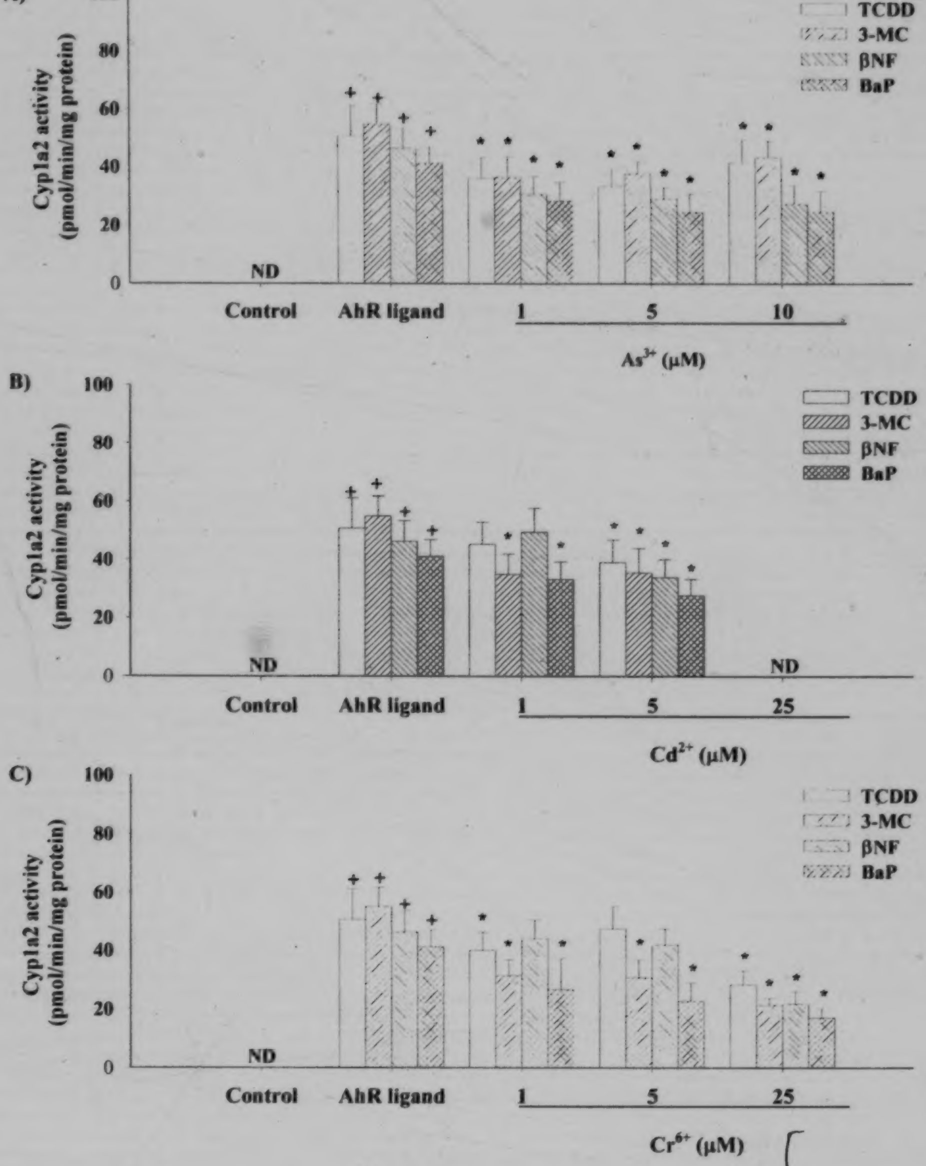

Figure 2.3. Effect of As^{3+} (A), Cd^{2+} (B), and Cr^{6+} (C) on the induction of Cyp1a2 (MROD) activity in murine hepatoma Hepa 1c1c7 by TCDD (0.1 nM), 3-MC (0.25 μM), βNF (10 uM), or BaP (1 μM). Values represent mean activity ± S.D. (n= 8). $^{+}p< 0.05$ compared to control; $*p<0.05$ compared to AhR ligand. ND: not detectable.

2.3.3.0. Effect of metals on constitutive and inducible Nqo1 and Gst a activities

As shown in Figure 2.4, TCDD, 3-MC, βNF, and BaP significantly induced Nqo1 activity in WT cells. However, βNF was more effective than the other three AhR ligands, causing a two-fold increase in activity, while TCDD was the least effective in inducing Nqo1 activity at the concentrations tested. The three metals alone also induced Nqo1 activity in WT cells in a dose-dependent manner. As^{3+} was the most potent inducer followed by Cd^{2+} and Cr^{6+} (Figure 2.4).

Not surprisingly, the three metals modulated the induction of Nqo1 activity by AhR ligands in a concentration, metal, and AhR ligand-dependent manner. Cr^{6+} caused a dose-dependent inhibition of Nqo1 activity induction by 3-MC, βNF, and BaP but had no significant effect on TCDD-mediated induction (Figure 2.4C).

Unlike Cr^{6+}, both As^{3+} and Cd^{2+} had paradoxical effects on AhR ligand-mediated induction of Nqo1 activity. As^{3+} inhibited Nqo1 activity induction by βNF at all concentrations tested although the inhibition was only significant at the 10 μM concentration (Figure 2.4A). With TCDD or 3-MC co-treatment, As^{3+} potentiated the induction of Nqo1 at all concentrations tested. However, when co-treated with BaP, As^{3+} enhanced ligand-mediated induction only at the 5 μM concentration, whereas the highest concentration (10 μM) had no significant effect. Similar to Cr^{6+}, Cd^{2+} also inhibited the induction of Nqo1 by βNF and BaP. However, similar to As^{3+}, Cd^{2+} potentiated Nqo1 activity induction by 3-MC, at all concentrations tested, although the induction was only significant at the lower doses (Figure 2.4B). Cd^{2+} did not seem to alter the induction of Nqo1 activity by TCDD.

With respect to Gst a, βNF was the only AhR ligand to significantly induce activity at the concentrations tested (Figure 2.5). All three metals alone, however, caused a significant increase in activity. However, Cr^{6+} induced activity at the 1 μM concentration but caused a dose-dependent inhibition at the 5 and 25 μM concentrations (Figure 2.5).

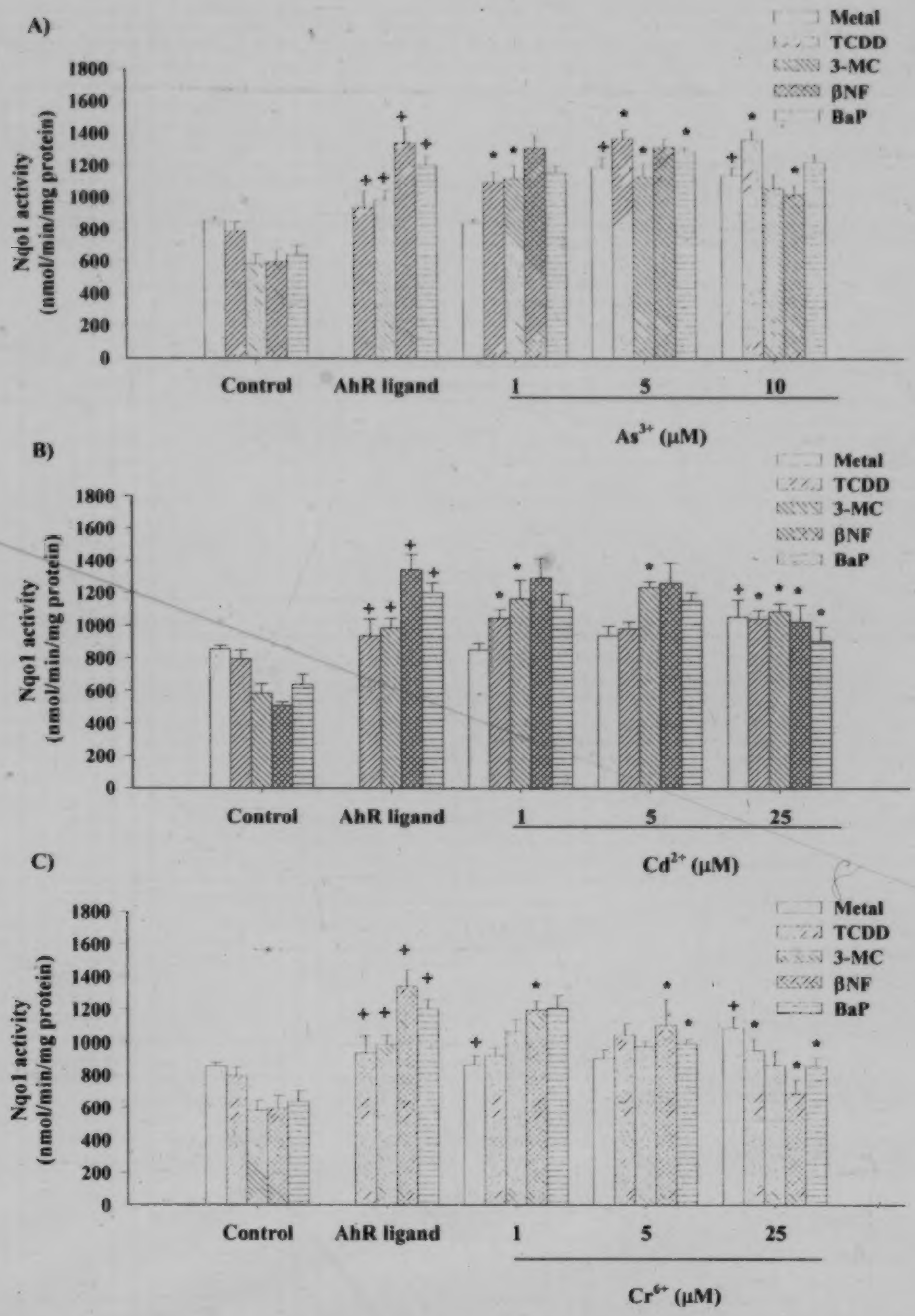

Figure 2.4. Effect of heavy metals on Nqo1 activity in Hepa 1c1c7 WT cells. Cells were treated for 24 h with increasing concentrations of the metals with and without one of the AhR ligands: TCDD (0.1 nM), 3-MC (0.25 µM), βNF (10 uM), or BaP (1 µM). Data are expressed as mean ± S.D. (*n*=6), $^{+}p<0.05$ compared to control; $*p<0.05$ compared to AhR ligand.

When the metals were co-treated with AhR ligands, As^{3+} and Cd^{2+} caused a dose-dependent increase in the Gst a catalytic activity (Figure 2.5A and 2.5B). In contrast, Cr^{6+} augmented Gst a activity with TCDD co-treatment only, by about 28% with all concentrations (Figure 2.5C). With BaP co-treatment however, Cr^{6+} 1 μM induced activity but there was a dose-dependent inhibition of Gst a activity at the 5 and 25 μM concentrations. Similarly, although Cr^{6+} 1 μM did not produce a significant change with 3-MC co-treatment, there was a dose-dependent inhibition of Gst a activity, compared to 3-MC treatment alone. Treatment of Cr^{6+} with βNF did not result in any significant changes, compared to βNF treatment alone.

2.3.4.0. Effect of metals on AhR-regulated genes in Hepa 1c1c7 C12 cells

To verify the role of the AhR in the regulation of AhR-regulated genes and to clarify its importance in modulating the effects of heavy metals, the same experiments were repeated on the C12 cell line. As with the WT cells, the basal levels of EROD and MROD activities were also not detectable. Also, none of the AhR ligands or metals, alone or in combination, caused induction of EROD or MROD activities.

As can be seen in Figure 2.6A, baseline Nqo1 activity in C12 cells was substantially lower than the baseline in WT cells, by up to 6.5-fold. Although C12 cells are not completely deficient in AhR, all four ligands did not have a significant effect on the activity of Nqo1, emphasizing the critical role of the AhR in the regulation of Nqo1. Furthermore, Cr^{6+} treatments alone, or with an AhR ligand, did not alter Nqo1 activity. However, both As^{3+} and Cd^{2+} caused a significant induction in Nqo1 activity, alone and when co-treated with an AhR ligand. As^{3+} and Cd^{2+} treatments alone caused 3.4- and 2.7-fold increases in Nqo1 activity, respectively, which was considerably higher than the induction observed when these metals were co-treated with AhR ligands. Thus, these two metals modulate Nqo1 activity in C12 cells by an AhR-independent mechanism.

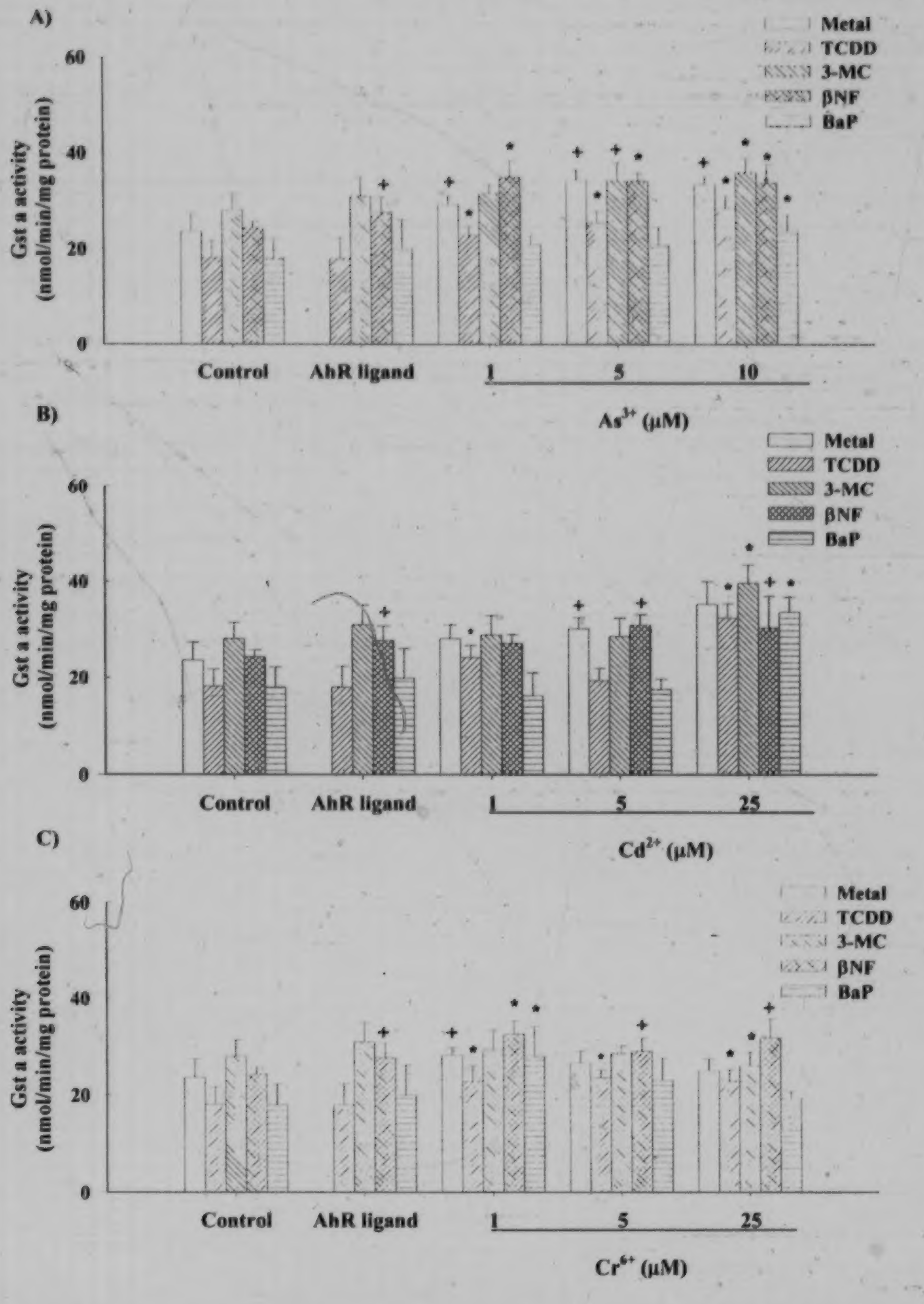

Figure 2.5. Effect of heavy metals on Gst a activity in Hepa 1c1c7 WT cells. Cells were treated for 24 h with increasing concentrations of the metals with and without one of the AhR ligands: TCDD (0.1 nM), 3-MC (0.25 μM), βNF (10 uM), or BaP (1 μM). Data are expressed as mean ± S.D. (n=6), $^{+}p<0.05$ compared to control; $*p<0.05$ compared to AhR ligand.

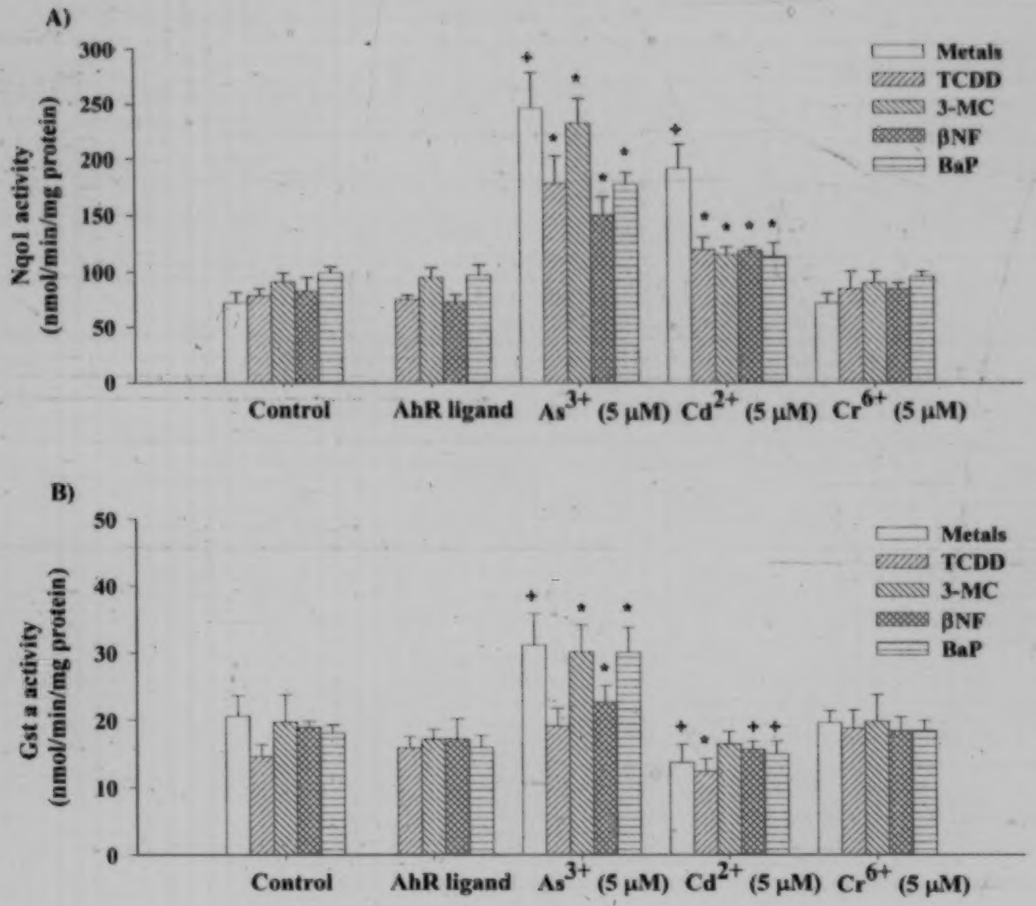

Figure 2.6. Effect of metals, with and without an AhR ligand, on Nqo1 (A) and Gst a (B) activities in Hepa 1c1c7 C12 cells. Cells were treated for 24 h with increasing concentrations of the metals with and without one of the the AhR ligands: TCDD (0.1 nM), 3-MC (0.25 μM), βNF (10 uM), or BaP (1 μM). Data are expressed as mean ± S.D. (*n*=6), $^{+}p<0.05$ compared to control; $*p<0.05$ compared to AhR ligand.

In contrast to Nqo1 activity, the difference between baseline Gst a activity in C12 and WT cells was minimal (Figure 2.6B). However, all four AhR ligands failed to produce a significant change in baseline activity. Nevertheless, As^{3+} treatment, whether alone or co-treated with an AhR ligand, also induced Gst a activity in C12 cells by an average of 1.5-fold. Surprisingly, however, Cd^{2+} decreased Gst a activity to below basal levels in all treatments. Cr^{6+} on the other hand had no discernable effect Gst a expression in C12 cells, whether treated alone or with an AhR ligand (Figure 2.6B).

2.3.5.0. Effect of metals on constitutive and inducible Cyp1a1 mRNA levels in WT cells

To determine whether modulation of Cyp1a1 enzyme activity is due to changes in mRNA levels, we investigated the effect of metals on Cyp1a1 mRNA expression in WT cells. As^{3+}, Cd^{2+}, or Cr^{6+} alone increased Cyp1a1 mRNA levels by 2.1-, 1.6-, and 1.7-fold, respectively, at the highest concentrations tested (Figure 2.7). Contradictory to the results obtained with the EROD and MROD assays, As^{3+} further potentiated the induction of Cyp1a1 mRNA mediated by AhR ligands, in a dose-dependent manner (Figures 2.8, 2.9, 2.10 and 2.11). Similarly, both Cd^{2+} and Cr^{6+} treatments augmented the induction of Cyp1a1 mRNA by βNF, BaP, TCDD and 3-MC. At the highest concentrations used in this study, Cr^{6+} was the most effective at enhancing the induction of Cyp1a1 mRNA by βNF and BaP while the induction by 3-MC and TCDD was potentiated best by As^{3+}. Of the four AhR ligands used in this study, the induction of Cyp1a1 mRNA by TCDD was the least affected by the three metals (Figure 2.8).

2.3.6.0. Effect of metals on constitutive and inducible Cyp1a1 protein levels

To assess whether the inhibitory effects of heavy metals on the inducible cyp1a1 activity were attributed to a pre- or post-translational mechanisms, quantitative measurement of Cyp1a1 protein using Western blot analysis was carried out. As expected, all AhR ligands tested significantly induced the Cyp1a1 protein level (Figure 2.12). Our results clearly showed that Cyp1a1 protein expression did not correlate well with the mRNA levels.

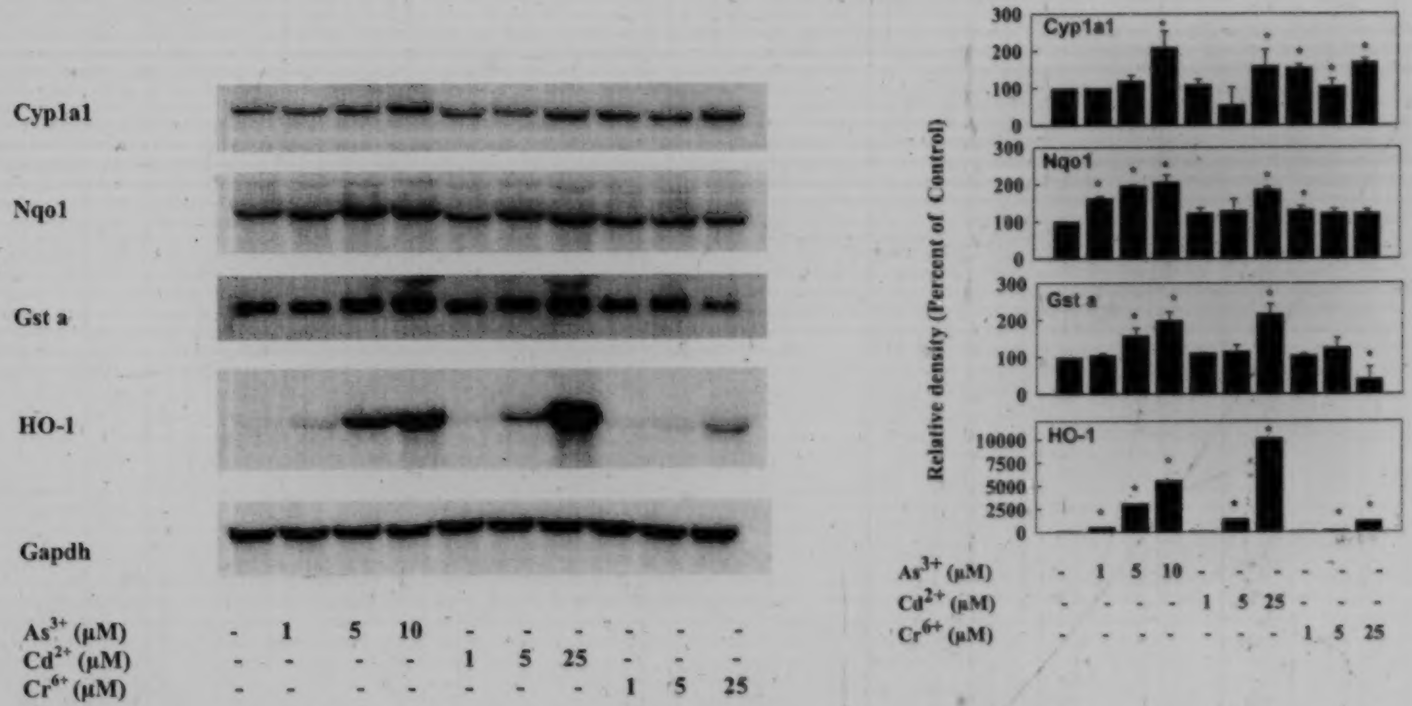

Figure 2.7. Effect of metals alone on Cyp1a1, Nqo1, Gst a, and HO-1 mRNA levels in Hepa 1c1c7 WT cells. Cells were treated with the metals in serum free medium and mRNA was extracted 6 h later using TRIzol reagent. Total RNA (20 µg) was separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a [^{32}P]-labeled cDNA. The blots were subsequently stripped and re-hybridized with a different cDNA probe. Northern blots were performed at least three times using different samples but only one representative result is shown. *p<0.05 compared to control.

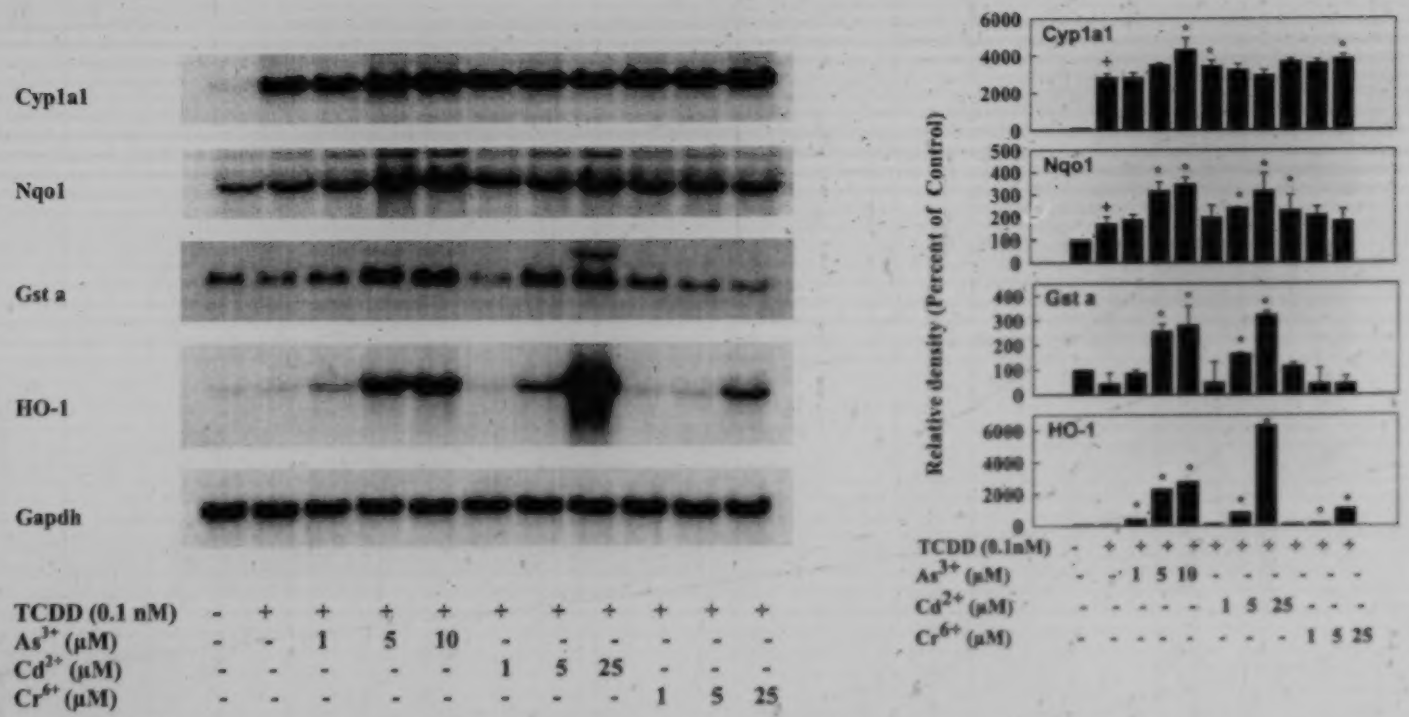

Figure 2.8. Effect of metals and TCDD on Cyp1a1, Nqo1, Gst a, and HO-1 mRNA levels in Hepa 1c1c7 WT cells. Cells were pre-treated with the metals in serum free medium 30 min before co-treatment with the AhR ligand. mRNA was extracted 6 h later using TRIzol reagent. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a [^{32}P]-labeled cDNA. The blots were subsequently stripped and re-hybridized with a different cDNA probe. Northern blots were performed at least three times using different samples but only one representative result is shown. $^{+}p<0.05$ compared to control; $^{*}p<0.05$ compared to AhR ligand.

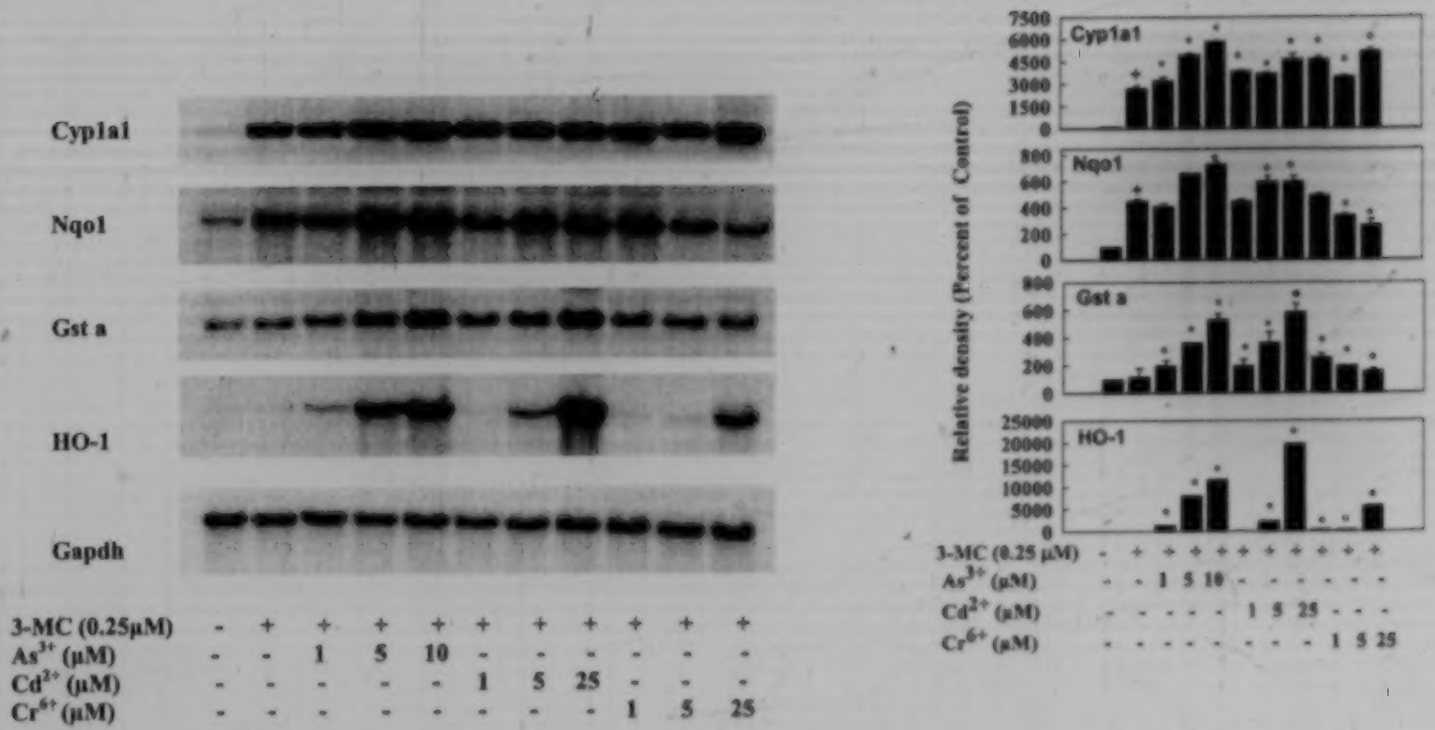

Figure 2.9. Effect of metals and 3-MC on Cyp1a1, Nqo1, Gst a, and HO-1 mRNA levels in Hepa 1c1c7 WT cells. Cells were pre-treated with the metals in serum free medium 30 min before co-treatment with the AhR ligand. mRNA was extracted 6 h later using TRIzol reagent. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a [^{32}P]-labeled cDNA. The blots were subsequently stripped and re-hybridized with a different cDNA probe. Northern blots were performed at least three times using different samples but only one representative result is shown. $^{+}p<0.05$ compared to control; $^{*}p<0.05$ compared to AhR ligand.

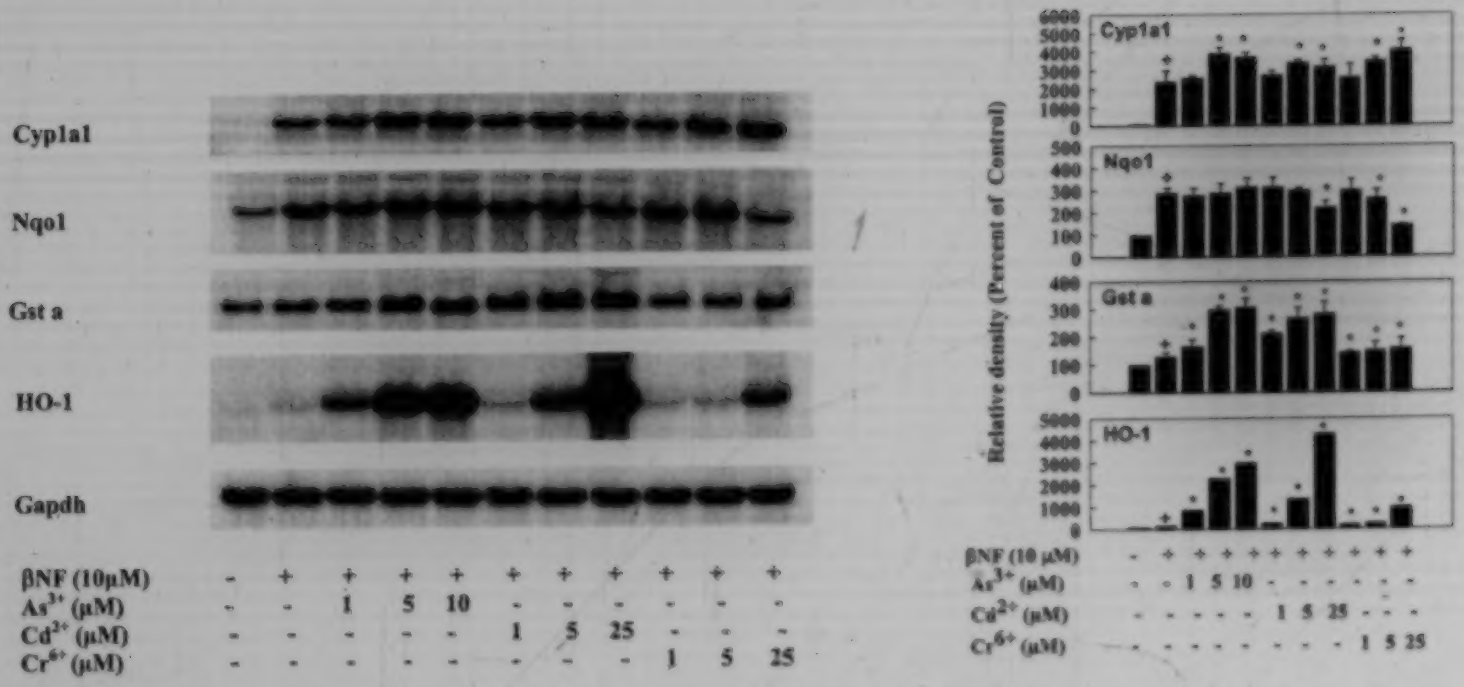

Figure 2.10. Effect of metals and βNF on Cyp1a1, Nqo1, Gst a, and HO-1 mRNA levels in Hepa 1c1c7 WT cells. Cells were pre-treated with the metals in serum free medium 30 min before co-treatment with the AhR ligand. mRNA was extracted 6 h later using TRIzol reagent. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a [^{32}P]-labeled cDNA. The blots were subsequently stripped and re-hybridized with a different cDNA probe. Northern blots were performed at least three times using different samples but only one representative result is shown. $^{+}p<0.05$ compared to control; $^{*}p<0.05$ compared to AhR ligand.

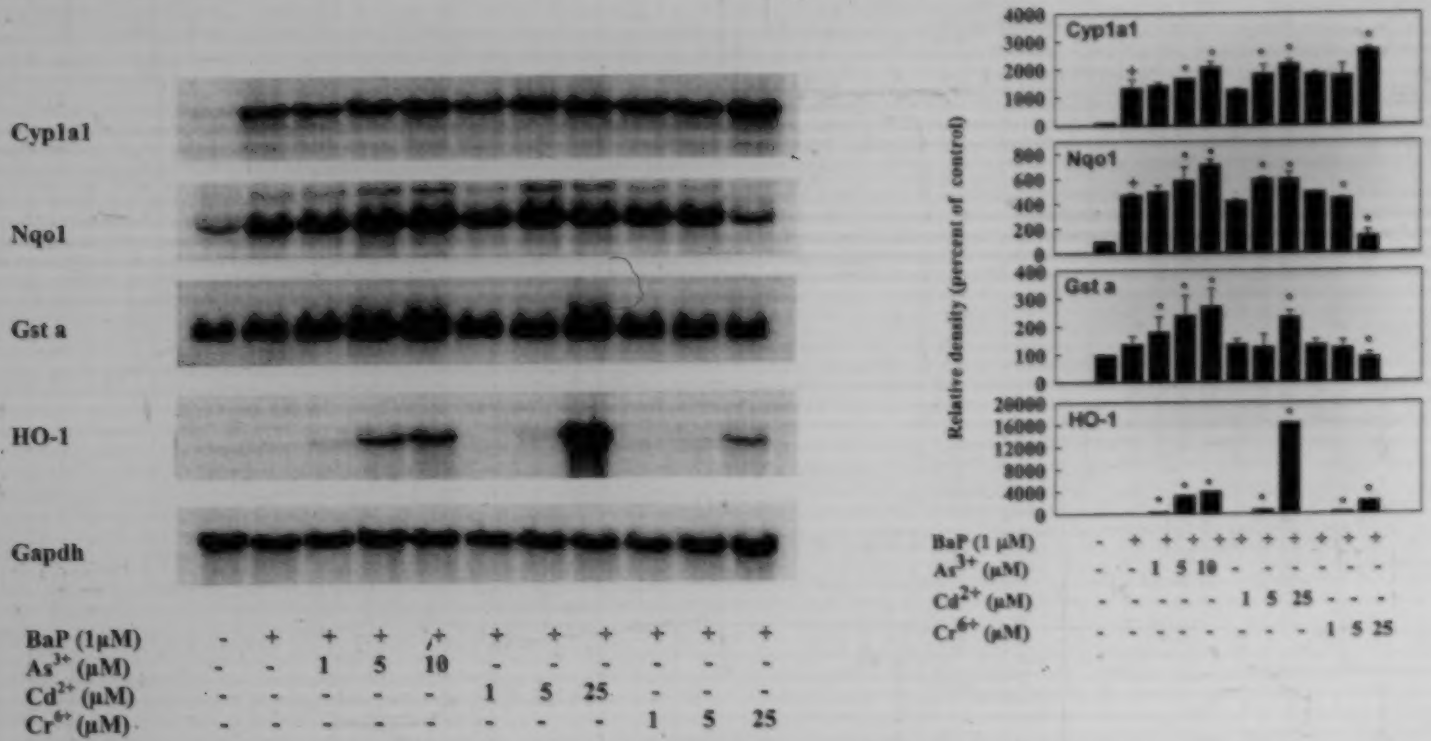

Figure 2.11. Effect of metals and BaP on Cyp1a1, Nqo1, Gst a, and HO-1 mRNA levels in WT cells. Cells were pre-treated with the metals in serum free medium 30 min before co-treatment with the AhR ligand. mRNA was extracted 6 h later using TRIzol reagent. Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a [^{32}P]-labeled cDNA. The blots were subsequently stripped and re-hybridized with a different cDNA probe. Northern blots were performed at least three times using different samples but only one representative result is shown. $^{+}p<0.05$ compared to control; $^{*}p<0.05$ compared to AhR ligand.

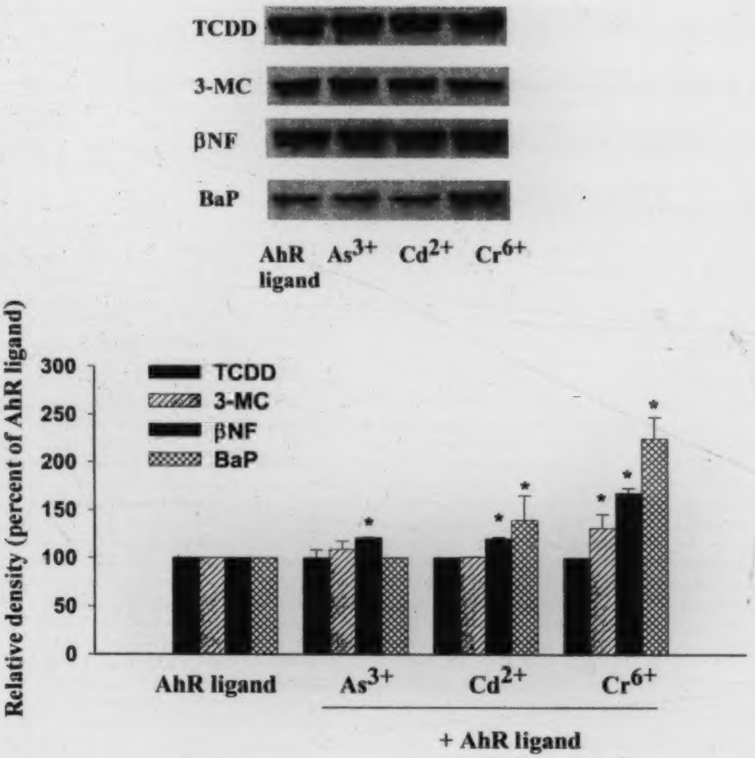

Figure 2.12. Effect of As^{3+} (5 μM), Cd^{2+} (5 μM) and Cr^{6+} (5 μM) on the induction of Cyp1a1 protein by AhR ligands in Hepa 1c1c7 WT cells. Cells were treated with a metal and one of the the AhR ligands: TCDD (0.1 nM), 3-MC (0.25 μM), βNF (10 μM), or BaP (1 μM). Cells were then harvested 24 h after treatment and the cellular homogenates were extracted. Proteins were resolved by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and sequentially incubated with a primary anti-mouse Cyp1a1 antibody and a peroxidase-conjugated IgG secondary antibody. Graph represents the average optical density (±S.D.) of bands from three different experiments, as a percent of AhR ligand. Only one representative blot is shown. *$p<0.05$ compared to AhR ligand.

Although Cr^{6+} potentiated the induction of Cyp1a1 protein when co-treated with 3-MC, βNF and BaP, Cd^{2+}enhanced Cyp1a1 protein induction by βNF and BaP, while the same effect was only obvious when As^{3+} was co-treated with βNF (Figure 2.12). Other treatments did not produce a significant decrease in protein expression, although all metals further potentiated Cyp1a1 mRNA expression by all AhR ligands. Interestingly, the induction of Cyp1a1 protein by TCDD was the least affected by the three metals (Figure 2.12). Cyp1a1 protein levels were not detectable when the cells were treated with metals alone.

2.3.7.0. Effect of metals on constitutive and inducible Nqo1 and Gst a mRNA levels

To further understand how metals alter the expression of Nqo1, we examined the effect of metal–AhR ligand co-treatments on Nqo1 mRNA expression in WT cells. Parallel to the results obtained in the activity studies, all four AhR ligands induced Nqo1 mRNA expression considerably when compared to their respective controls (Figures 2.8-2.11). βNF, BaP, 3-MC, and TCDD induced mRNA expression by 4, 5, 3, and 1.5-fold, respectively (Figures 2.8-2.11). As^{3+} or Cd^{2+} alone also induced Nqo1 mRNA expression by two-fold at the highest concentrations tested (Figure 2.7). On the other hand, Cr^{6+} treatment alone caused only a minor induction in Nqo1 mRNA expression in a non-dose-dependent manner. When co-treated with AhR ligands, Cr^{6+} and As^{3+} had consistent effects on Nqo1 mRNA induction by AhR ligands (Figures 2.8-2.11). Cr^{6+} caused a dose-dependent decrease in Nqo1 mRNA levels induced by βNF, BaP, and 3-MC, which is comparable to the effect of Cr^{6+} on Nqo1 activity. Also, Cr^{6+} potentiated Nqo1 mRNA induction by TCDD at the lower concentrations but failed to do so at the highest concentration.

Unlike Cr^{6+}, As^{3+} and Cd^{2+} potentiated Nqo1 mRNA induction by TCDD, 3-MC, and BaP in a dose-dependent manner (Figures 2.8, 2.9 and 2.11). On the other hand, As^{3+} did not modulate βNF-mediated induction of Nqo1 mRNA although Cd^{2+} caused a dose-dependent decrease in mRNA levels (Figure 2.10).

Our findings from the Gst a activity assays correlate well with the analysis of mRNA expression. As can be seen in Figure 2.10, βNF was the only AhR ligand to cause a noticeable increase in Gst a mRNA levels (1.4-fold induction). However, As^{3+} or Cd^{2+} treatments alone, or treated with AhR ligands, increased mRNA levels considerably and in a dose-dependent manner (Figure 2.7). In contrast to the other metals, but consistent with the activity results, Cr^{6+} treatment alone caused a dose-dependent decrease in Gst a mRNA levels.

2.3.8.0. Effect of metals on HO-1 mRNA expression in WT cells

As a first attempt to explain the mechanism by which heavy metals alter the expression of AhR-regulated genes, we measured HO-1 mRNA expression in WT and C12 cells. Of the four ligands used in this study, only βNF induced HO-1 mRNA, although the induction was minimal (Figures 2.8-2.11). In WT cells, metal treatments alone and co-treatments with AhR ligands resulted in a dose-dependent increase in HO-1 mRNA levels (Figures 2.7-2.11). Of the three metals, Cd^{2+} was the most potent in inducing HO-1 mRNA, followed by As^{3+}, while Cr^{6+} was the weakest inducer (Figure 2.7).

2.4.0.0. Discussion

There is an abundance of evidence on the carcinogenicity and mutagenicity of heavy metals and AhR ligands. However, many of these studies have failed to examine the carcinogenic risk of mixtures of these compounds which is of vital importance as these toxins exist as co-contaminants in the environment. There are various mechanisms by which AhR ligands exert their genotoxic effects and heavy metals may alter any one, or all, of these mechanisms. We are undertaking extensive studies to investigate the effect of metals on one pathway of AhR ligand mediated carcinogenesis. Here, we report the ability of As^{3+}, Cd^{2+}, and Cr^{6+} to influence the bioactivation of AhR ligands by phase I drug metabolizing enzymes and the inactivation of their reactive intermediates by phase II drug metabolizing enzymes.

Concentrations of the metals were chosen based on ranges reported earlier in the literature (Maier et al., 2000, Vakharia et al., 2001a and Vakharia et al., 2001b). The

MTT assay was used to ensure that the doses chosen did not alter the viability of WT cells. The effect of metals on the viability of C12 cells was also performed. It is of interest to note that the metals exerted a more toxic response on C12 cells, in comparison to their effect on WT cells, as determined by the MTT assay. All three metals, As^{3+}, Cd^{2+}, and Cr^{6+}, alter cell growth and induce apoptosis (Blankenship et al., 1994, el Azzouzi et al., 1994 and Ochi et al., 1996). Since the AhR is responsible for the regulation of not only drug metabolizing enzymes but also of other genes that influence growth, differentiation, and apoptosis (Okey et al., 1994), it is not surprising that the AhR deficient cells are more sensitive to the toxic effects of the metals. Nevertheless, the lowest metal doses that induced changes in enzyme activities in WT cells and yet did not affect the integrity of at least 60% of C12 cells were chosen to study enzyme expression in the AhR deficient cells.

The results presented in this study demonstrate the ability of As^{3+}, Cd^{2+}, and Cr^{6+} to inhibit the induction of Cyp1a1 activity by AhR ligands. Although one study showed that As^{3+} had no discernable effect on inducible Cyp1a1 activity in murine hepatoma cells (Maier et al., 2002), nevertheless, our results are in general agreement with previous reports of metal effects on Cyp1a1 activity (Jacobs et al., 1999, Vakharia et al., 2001b and Spink et al., 2002). As^{3+} and Cd^{2+} inhibited AhR ligand-mediated induction of CYP1A1 activity and protein levels in HepG2 cells (Vakharia et al., 2001a). As^{3+} also decreased the 3-MC mediated induction of CYP1A in cultured chick (Jacobs et al., 1998) and rat hepatocytes (Jacobs et al., 1999) and the BaP-mediated induction of CYP1A1 and 1B1 in T-47D human breast cancer cells (Spink et al., 2002).

Surprisingly, in the current study, all three metals enhanced Cyp1a1 mRNA levels. This is in disagreement with the EROD activity results and with most previous reports showing either no effect on CYP1A mRNA induction (Maier et al., 2000 and Spink et al., 2002) or a decrease in mRNA induced levels (Jacobs et al., 1999). The results provided here present us with the first evidence that the modulation of Cyp1a1 enzyme activity is likely due to post-transcriptional mechanism(s). There are several explanations for the enhancement of mRNA levels by the various heavy metals. Metals may cause the breakdown of cellular products and the production of non-classical AhR ligands, thus inducing Cyp1a1 mRNA expression (Tchounwou et al., 2001). Rather than increasing the transcription, the metals may have also altered mRNA levels by stabilizing the mRNA transcripts.

Complementary to the mRNA results, treatment with metals either potentiated or had no discernable effect on the protein expression of Cyp1a1, when compared to the level of protein induced by AhR ligands alone. Thus, post-translational mechanism(s) are responsible for the inhibition of Cyp1a1 activity induction by metals. One mechanism by which metals may inhibit CYP450 activity is through the induction of HO-1. In our study, all three metals increased HO-1 mRNA levels in a dose-dependent manner, which parallels the pattern of a dose-dependent inhibition of EROD and MROD activities. However, the induction of HO-1 does not explain why the weakest HO-1 inducer, Cr^{6+}, was the most potent at inhibiting 3-MC- and TCDD- mediated induction of EROD activity or the lack of dose dependency with As^{3+} treatments. Furthermore, the addition of increasing concentrations of heme did not prevent As^{3+} mediated decreases in CYP2B1/2 or CYP1A1 activities in rat hepatocyte cultures (Jacobs et al., 1999). Hence, although HO-1 induction may contribute to the inhibition of EROD and MROD activities by the metals, there must other interplaying mechanisms.

Interestingly, As^{3+} has been shown to react with cysteine residues of proteins, causing loss of protein function (Kapahi et al., 2000). Alternatively, metals may down-regulate CYP450 function post-translationally via the production of reactive oxygen species (ROS). The production of ROS by As^{3+}, Cd^{2+}, and Cr^{6+} has been previously reported (Pourahmad et al., 2003). ROS reduced the catalytic activities of CYP1A1/1A2 without decreasing their protein content (El-Kadi et al., 2000). Thus, we are postulating that metal-mediated ROS production may inactivate CYP450 enzymes by interacting with the heme Fe^{2+} in the enzymes or by acting as second messengers in a signaling pathway leading to the phosphorylation of CYP450 (Lowe et al., 1998).

The coordinated regulation of phase I and phase II enzymes is an important determinant of toxicant fate since phase II enzymes protect the cell against electrophiles which are by-products of the phase I metabolism of some AhR ligands. Two phase II enzymes which are under partial control of the AhR are Nqo1 and Gst a. Hence, we undertook studies to examine whether metals modulated the induction of these enzymes by AhR ligands.

In the current study, the four AhR ligands significantly enhanced Nqo1 activity in WT cells. However, this enzyme activity was less inducible than Cyp1a1, an effect which was especially noticeable with TCDD treatments. This result is not surprising however, due to early studies which reported that some phase II enzymes were less inducible by TCDD than CYP1A1 and CYP1A2 (Vanden Heuvel et al., 1994).

Metals differentially altered Nqo1 induction in WT cells by AhR ligands in a ligand- and metal- specific manner. The ability of As^{3+} and Cd^{2+} to induce Nqo1 (Falkner et al., 1993b) and Gst a (Talalay et al., 1995) has been previously documented. In this study, As^{3+} and Cd^{2+} potentiated the induction of Nqo1 and Gst a activities, corresponding to increased mRNA levels.

On the other hand, Cr^{6+} inhibited Nqo1 activity and mRNA induction by TCDD, 3-MC, βNF and BaP, likely by directly interacting with DNA (Klein, 1996). The differential effect of Cr^{6+} on inducible gene expression seems to be a global one as the same observations have been reported with non-AhR-regulated genes (Hamilton and Wetterhahn, 1989). Recently, it has been shown that Cr^{6+} at a high concentration inhibits the catalytic activities and mRNA expression of Cyp1a1, Nqo1 and Gst a (Wei et al., 2004). The mechanism by which Cr^{6+} inhibited AhR-regulated genes is by blocking the recruitment of the coactivator protein p300 and its associated histone acetylation.

Here, we have shown that As^{3+} and Cd^{2+} may decrease the carcinogenic and mutagenic effects of AhR ligands by decreasing the induction of phase I and enhancing the induction of phase II enzymes. The effect of Cr^{6+} on the otherhand is not clear as we have found it decreased the activities of both phase I and II enzymes. Future studies to determine the mechanisms by which heavy metals alter the function of these enzymes may explain the differential effect of metals on the expression of AhR-regulated genes and their induction by AhR ligands.

2.5.0.0.0 References

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3.0.0.0. Chapter 3.0

TRANSCRIPTIONAL ACTIVATION AND POSTTRANSCRIPTIONAL MODIFICATION OF CYP1A1 BY ARSENITE, CADMIUM, AND CHROMIUM.

A version of this chapter has been published: Elbekai, R.H. and El-Kadi, A.O., 2007 *Toxicol Lett.* **172**, pp. 106-119.

3.1.0.0. Introduction

We have previously shown that the heavy metals, arsenite (As^{3+}), cadmium (Cd^{2+}), and chromium (Cr^{6+}) inhibit AhR ligand-mediated induction of Cyp1a1 activity but potentiate the induction of Nqo1 and Gst a activities (chapter 2). Nonetheless, the molecular mechanisms involved in the modulation of Cyp1a1 expression need to be further investigated. Understanding putative mechanisms involved in the modulation of Cyp1a1 expression is pivotal in enhancing our knowledge of the regulation of Cyp1a1 and in defining the exact role of AhR ligands and metals in carcinogenicity and mutagenicity.

The objective of this study was to investigate the molecular mechanisms involved in the modulation of Cyp1a1 expression,by heavy metals at each step of the AhR signal transduction pathway. To investigate these mechanisms, we determined the time course of the induction of Cyp1a1 mRNA by heavy metals and the role of transcriptional activation, specifically through the AhR, in the increase in Cyp1a1 mRNA. In addition, we determined the effect of heavy metals on Cyp1a1 mRNA degradation. Looking at the posttranslational modulation by heavy metals, we investigated the effect of heavy metals on Cyp1a1 protein stability and function and studied the possible involvement of heme oxygenase 1 (HO-1) in the decrease in Cyp1a1 function. In the current study we used a single concentration from each metal, which were 5 μM, 5 μM, and 25 μM for As^{3+}, Cd^{2+}, and Cr^{6+}, respectively. The concentrations of metals used in the current study were chosen after determining the ability of a range of concentrations to modulate *Cyp1a1* gene expression without significantly affecting Hepa 1c1c7 cell viability (chapter 2). Our investigations provide the first evidence for the involvement of transcriptional and posttranscriptional mechanisms in the modulation of Cyp1a1 expression by As^{3+}, Cd^{2+}, and Cr^{6+}.

3.2.0.0. Materials and methods

3.2.1.0. Materials

Sodium arsenite, cadmium chloride, chromium trioxide, cycloheximide (CHX), 7-ethoxyresorufin, fluorescamine, anti-goat IgG peroxidase secondary antibody, and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent, and the random primers DNA labeling system were purchased from Invitrogen (Grand Island, NY). Actinomycin-D (Act-D) and carbobenzoxy-l-leucyl-l-leucyl-leucinal (MG-132) were purchased from Calbiochem (San Diego, CA). Chemiluminescence western blotting detection reagents and hybond-N-nylon membranes were from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). Cyp1a1 goat polyclonal primary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). α-^{32}PdCTP and γ^{32}PATP were supplied by the DNA Core Services Laboratory, University of Alberta. All other chemicals were purchased from Fisher Scientific (Toronto, Ont.).

3.2.2.0. Cell culture

Hepa 1c1c7 cell lines, generously provided by Dr. O. Hankinson at the University of California (Los Angeles, CA), were maintained in Dulbecco's modified Eagle's medium (DMEM), without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamycin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified incubator.

3.2.3.0. Chemical treatments

Cells were treated in serum free medium with 1 nM TCDD, 5 μM As^{3+}, 5 μM Cd^{2+}, or 25 μM Cr^{6+} as described in figure legends. TCDD was dissolved in dimethylsulfoxide

(DMSO) and maintained in DMSO at −20 °C until use. The metals were prepared freshly in double de-ionized water. In all treatments, the concentration of double de-ionized water in treatment medium did not exceed 0.5% (v/v) while the DMSO concentration did not exceed 0.05% (v/v).

3.2.4.0. RNA extraction and analysis

After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen). Northern blot analysis of total RNA was performed as described elsewhere (chapter 2). The cDNA probes for mouse Cyp1a1, HO-1, and Gapdh mRNA were generously provided by Dr. John R. Bend (University of Western Ontario, London, Ont.). All probes were α^{32}P-labeled by the random primer labeling method according to the manufacturer's (Invitrogen) instructions.

3.2.5.0. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from Hepa 1c1c7 cells, treated for 2 h with vehicle, 5 nM TCDD, or 10 µM As^{3+}, 25 µM Cd^{2+}, or 25 µM Cr^{6+}, using the method of Denison et al. (2002). Also, guinea pig hepatic cytosol, generously provided by Dr. M.S. Denison (University of California, Davis, CA), was incubated with the test compounds for a final concentration of 20 nM TCDD, 10 µM As^{3+}, 25 µM Cd^{2+}, or 25 µM Cr^{6+}, for 2 h at 20 °C. Protein concentrations for the nuclear and cytosolic extracts were determined using the method of Lowry (Lowry et al., 1951), using bovine serum albumin as a standard. To visualize the ability of each of the heavy metals to induce the transformation and subsequent DNA binding of the AhR, a complementary pair of synthetic oligonucleotides containing the sequence 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCA-3′, corresponding to the XRE binding site, were synthesized and radiolabeled with γ^{32}PATP as previously described (Denison et al., 2002) and used as a DNA probe in all experiments. Binding reactions using aliquots of 120 µg cytosolic or 20 µg nuclear extracts and excess radiolabeled oligonucleotides were allowed to proceed for 15 min at 20 °C in a buffer containing

1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 25 mM HEPES, 400 ng to 2 μg poly(dI–dC), and 0.4–0.8 mM KCl. To determine the specificity of binding to the oligonucleotide, a 100-fold molar excess of unlabeled XRE probe was added to the binding reaction prior to addition of the γ^{32}P-labeled probe. Protein-DNA complexes were separated under non-denaturing conditions on a 4% polyacrylamide gel using 1× TBE (90 mM of Tris borate, 90 mM of boric acid, 4 mM of EDTA) as a running buffer. The gels were dried and the protein–DNA complexes were visualized by autoradiography.

3.2.6.0. Transient transfection and luciferase assay

Cells grown for 24 h in 6-well plates were transiently transfected using lipofectamine with the XRE-luciferase reporter plasmid pGudLuc1. Briefly, cells were incubated with DNA–lipofectamine complexes in serum-free, antibiotics-free medium. After at least a 18 h incubation period, cells were treated with vehicle, 5 nM TCDD, 10 μM As^{3+}, 25 μM Cd^{2+}, or 25 μM Cr^{6+} in fresh serum-free medium for 24 h before harvesting for luciferase assay. Luciferase activities were analyzed in 100-μl cell extracts with the The Luciferase Assay System (Promega) on a TD-20/20 luminometer (Turner BioSystems, Sunnyvale CA). Luciferase activity is reported as emitted light per well as a percent of control, vehicle treated cells.

3.2.7.0. Cyp1a1 mRNA stability

The half-life of Cyp1a1 mRNA was analyzed by an Act-D-chase assay. Cells were pre-treated with 1 nM TCDD for 12 h. Cells were then washed and incubated with 5 μg/ml Act-D, to inhibit further RNA synthesis, immediately before treatment with As^{3+}, Cd^{2+}, or Cr^{6+}. Total RNA was extracted at 0, 1, 3, 6, and 12 h after incubation with the metals. Northern blot analysis was performed followed by hybridization first with the Cyp1a1 probe and then with the Gapdh probe. Band densities were quantified using the ImageJ image processing program (National Institute of Mental Health, Bethesda, MD). The mRNA half-life values were determined from semilog plots of integrated densities versus time.

3.2.8.0. Cyp1a1 protein stability

The half-life of Cyp1a1 protein was analyzed by the CHX-chase assay. Cells were pre-treated with 1 nM TCDD for 24 h. Cells were then washed and incubated with 10 µg/ml CHX, to inhibit further protein synthesis, immediately before treatment with As^{3+}, Cd^{2+}, or Cr^{6+}. Cells were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µl/ml of protease inhibitor cocktail, at 0, 1, 3, 6, 12, and 24 h after incubation with the metals. The cellular homogenates were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Proteins (25 µg) were separated by 10% SDS-PAGE bis-acrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane as described previously (chapter 2). After blocking over-night in blocking buffer, protein blots were incubated with a primary polyclonal goat anti-mouse Cyp1a1 antibody for 2 h at room temperature. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody was then carried out in blocking buffer for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (GE Healthcare Life Sciences). Band densities were also then quantified using the ImageJ image processing program and the protein half-life values were determined from semilog plots of integrated densities versus time.

3.2.9.0. Determination of Cyp1a1 activity

The Cyp1a1-dependent 7-ethoxyreorufin-*O*-deethylase (EROD) activity was performed on intact, living cells as described previously (chapter 2). Enzymatic activity was normalized for cellular protein content which was determined using a modified fluorescent assay (chapter 2).

3.2.10.0. Determination of total cellular heme content

Cellular heme content was determined by a modification of the method of Ward (Ward et al., 1984). After a 24 h incubation period with 1 nM TCDD in the presence and absence of As^{3+}, Cd^{2+}, or Cr^{6+}, cells were pelleted and boiled in 2.0 M oxalic acid for 30 min followed by resuspension in cold phosphate buffered saline (PBS) and centrifugation at 14,000 × *g* for 15 min. The supernatant was then removed and the fluorescence of protoporphyrin IX was assayed using the Eclipse fluorescence spectrophotometer (Varian Australia PTY LTD., Australia) using an excitation wavelength of 405 nm and an emission wavelength of 600 nm. Background was determined by measuring the fluorescence in the absence of cells. Cellular heme content was calculated as a percent of serum-free medium treated cells following normalization of cellular heme content with cellular protein, which was determined using the method of Lowry (Lowry et al., 1951).

3.2.11.0. Statistical analysis

All results are presented as mean ± S.E.M. The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc comparison. The differences were considered significant when $P < 0.05$.

3.3.0.0. Results

In order to dissect the various molecular mechanisms involved in the regulation of *Cyp1a1* gene expression, the effect of heavy metals on each step of the AhR-signal transduction pathway was analyzed. For this purpose, we first determined the effect of metals on the expression of Cyp1a1 mRNA. We then explored the effect of the metals on the Cyp1a1 protein expression and function.

3.3.1.0. Effect of heavy metals on the time-dependent induction of Cyp1a1 mRNA

The time course of *Cyp1a1* gene expression was determined at various time points up to 24 h after treatment with a single concentration of TCDD and/or the metals. Each of the metals alone clearly produced a time-dependent increase in Cyp1a1 mRNA levels which was visible as early as 3 h following treatment of Hepa 1c1c7 cells with the metals (Figure 3.1A–C). As^{3+}, the most potent Cyp1a1 mRNA inducer at the concentrations used, caused a maximal 18-fold induction in Cyp1a1 mRNA 3 h after treatment (Figure 3.1A). Although this induction was maintained at 6 h following treatment, there was a three fold drop in the induction of Cyp1a1 mRNA by As^{3+}. Similarly, Cd^{2+}-induced Cyp1a1 mRNA reached a maximum at 3 h, followed by a decrease at 6 h. In contrast, the maximal induction due to Cr^{6+} was comparatively delayed, reaching a maximum 15-fold increase at 6 h. Interestingly, with all metals, there was a significant drop in Cyp1a1 mRNA at 12 h after treatment (Figure 3.1A–C). Gapdh mRNA, which was used as a loading control, remained stable over the time period of the study.

The profile of Cyp1a1 induction by TCDD is also shown in Figure 3.1. While there was no discernable induction of Cyp1a1 mRNA at 1 h post TCDD treatment, the expression significantly increased by 3 h, reaching a maximum at 6–12 h post-treatment (Figure 3.1). At 24 h, TCDD produced a significant increase in Cyp1a1 mRNA (12-fold induction), albeit it was 40% less than maximally attained levels. To determine the effect of the heavy metals on the TCDD-mediated time-dependent induction of Cyp1a1 mRNA, Hepa 1c1c7 cells were co-treated with TCDD and one of the metals. In parallel to their effect on the constitutive expression of Cyp1a1 mRNA, As^{3+} and Cd^{2+} significantly potentiated the induction of Cyp1a1 mRNA by TCDD at 3–6 h post-treatment. At 12–24 h post-treatment, As^{3+} did not significantly alter the level of TCDD-induced Cyp1a1 mRNA, although Cd^{2+} decreased the inducible expression at 12 h while markedly potentiating the induction at 24 h. In contrast to its effect on the constitutive expression of Cyp1a1 mRNA, Cr^{6+} significantly inhibited the TCDD-mediated time-dependent induction of Cyp1a1 mRNA at all measured time points (Figure 3.1C).

3.3.2.0. Transcriptional activation of the Cyp1a1 gene by heavy metals

To examine the role of transcriptional mechanisms in the induction of Cyp1a1 mRNA by heavy metals, a transcription inhibitor was used to prevent *de novo* RNA synthesis. Act-D, a blocker of DNA-dependent RNA synthesis, diminished constitutively expressed

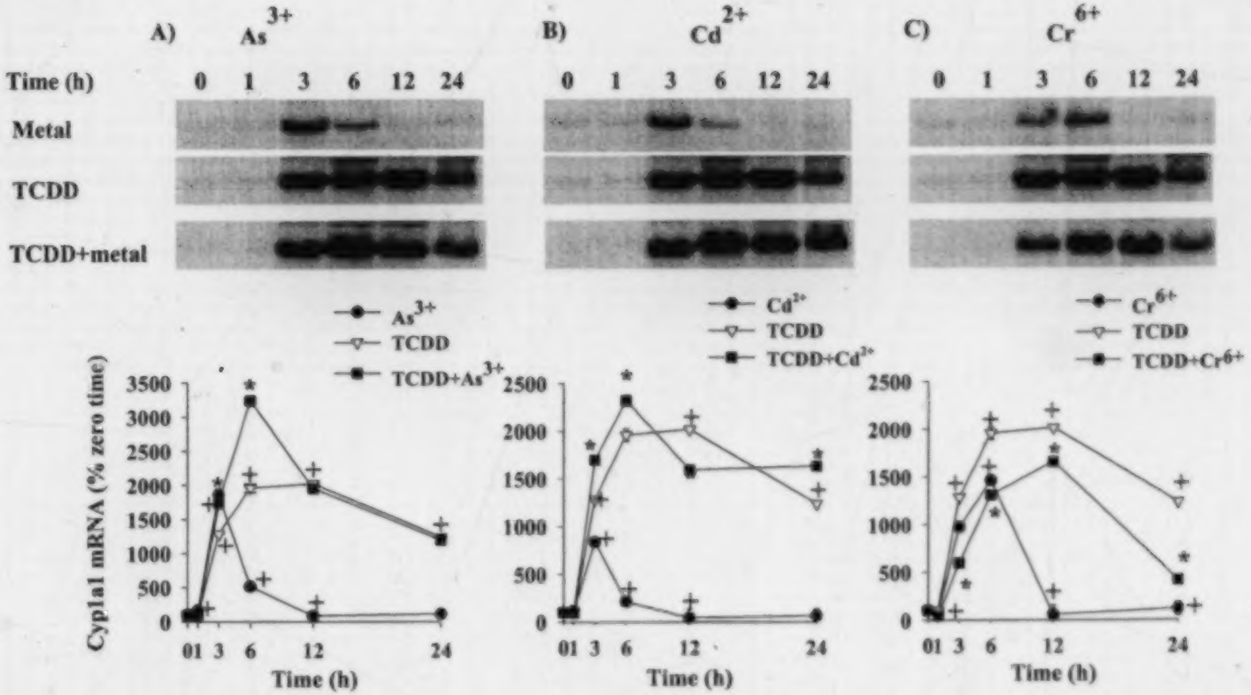


Figure 3.1. Time-dependent effects of heavy metals on the constitutive and inducible Cyp1a1 mRNA levels. Cells were treated with 5 μM As^{3+}, 5 μM Cd^{2+}, or 25 μM Cr^{6+} in the presence or absence of 1 nM TCDD for the time points indicated. Total cellular RNA was collected at 0, 1, 3, 6, 12, and 24 h after treatment using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to hybond-N nylon membranes, and hybridized with $\alpha^{32}P$-labeled Cyp1a1 and Gapdh cDNA probes. Graphs represent Gapdh-standardized densitometric readings for Cyp1a1 mRNA expressed as a percent of Cyp1a1 mRNA levels in the presence of vehicle only (mean ± S.E.M., $n = 3$). One of three representative experiments is shown. $+p < 0.05$, compared to control; $*p < 0.05$, compared to respective TCDD treatment.

Cyp1a1 mRNA in vehicle-treated cells (Figure 3.2, lane 2). Exposure to Act-D also efficiently blocked the induction of Cyp1a1 mRNA by As^{3+}, Cd^{2+}, and Cr^{6+} (Figure 3.2, lanes 4, 6, and 8, respectively). The inhibition of metal-mediated induction of Cyp1a1 mRNA by Act-D was similar to the effect of this transcription inhibitor on the TCDD-mediated response (Figure 3.2, lane 10). With what is known about the transcriptional regulation of the *Cyp1a1* gene, it is likely that metal-mediated Cyp1a1 mRNA induction is due, at least in part, to transcriptional mechanisms.

3.3.3.0. The role of the AhR in the transcriptional activation of the Cyp1a1 gene by heavy metals

To determine the mechanism by which metals increase *Cyp1a1* gene transcription, we studied potential ability of the heavy metals to bind to and stimulate AhR transformation and DNA binding. We first examined the ability of each of the metals to directly activate the AhR *in vitro*. Incubation of guinea pig hepatic cytosol with TCDD induced the formation of a DNA–protein complex, representing the transformed AhR bound to the XRE (Figure 3.3A). In contrast, neither As^{3+}, Cd^{2+}, nor Cr^{6+} induced the formation of a DNA–protein complex (Figure 3.3A). However, Cd^{2+} and Cr^{6+}, but not As^{3+}, produced an inducible protein-DNA complex, *in vivo*, that migrated to the same position as that produced by TCDD, albeit with lower potency (Figure 3.3B). Hence, it is apparent that Cd^{2+} and Cr^{6+} activate the AhR indirectly.

To further confirm the role of the AhR in metal-mediated increase in Cyp1a1 transcription, Hepa 1c1c7 cells transiently transfected with the XRE-driven luciferase reporter gene were used to study AhR-dependent transcription activation. In agreement with the EMSA results, all three metals induced luciferase activity, confirming the role of the AhR in metal-mediated Cyp1a1 mRNA induction. As shown in Figure 3.4, a 4-, 7-, and 2.5-fold induction of luciferase activity was observed in cells treated with As^{3+}, Cd^{2+}, and Cr^{6+}, respectively.

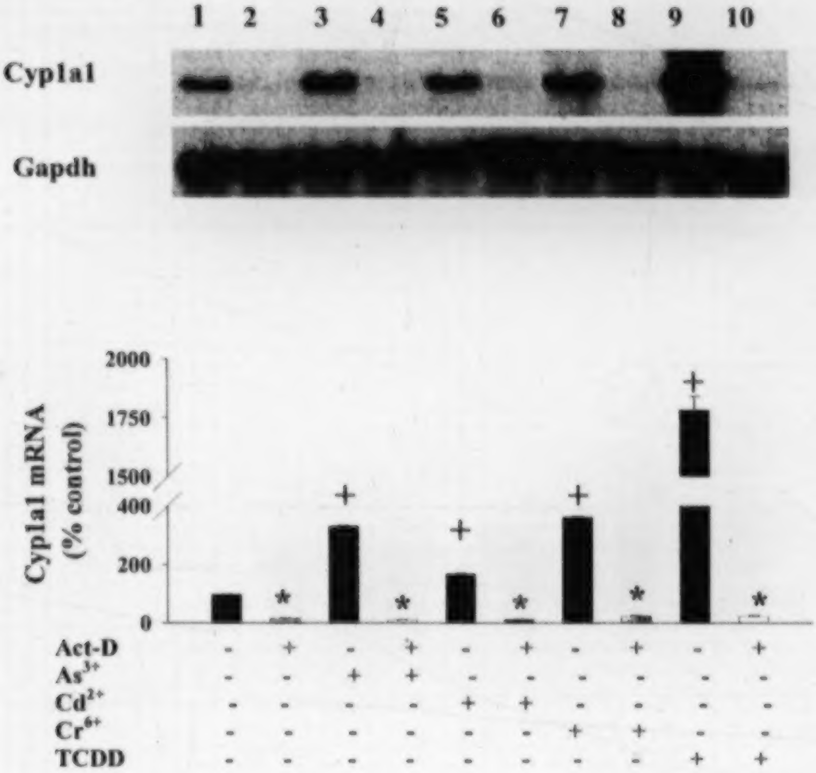
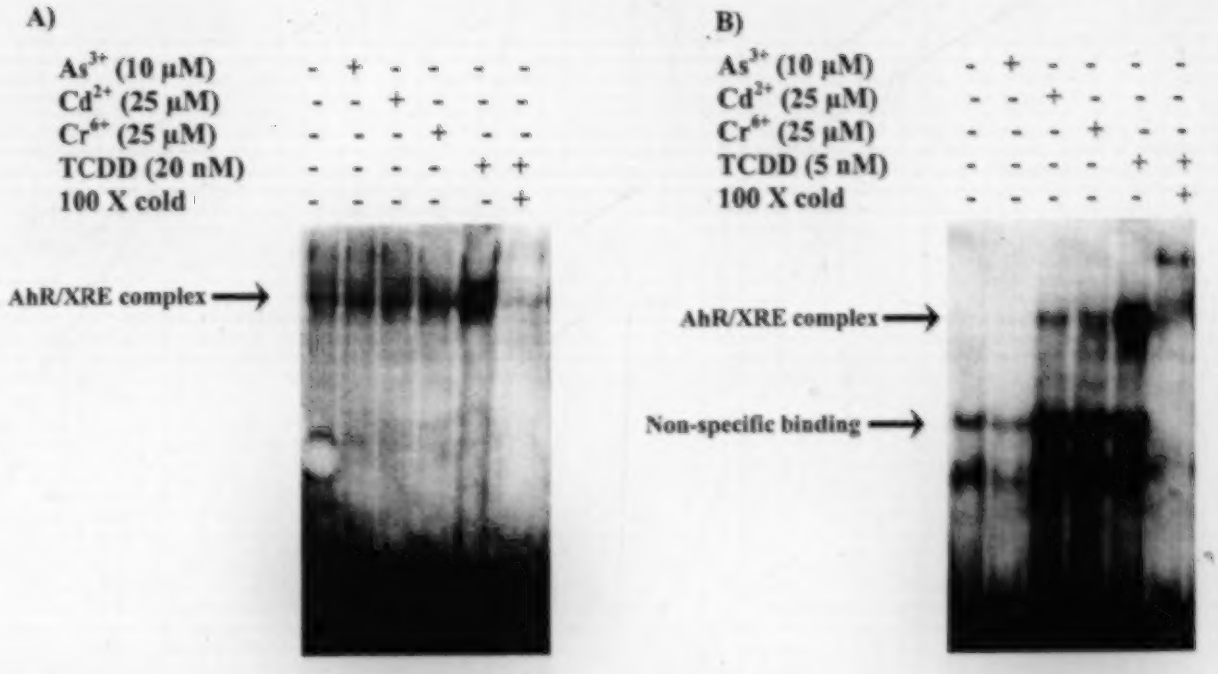


Figure 3.2. Effect of actinomycin-D on metal-mediated induction of Cyp1a1 mRNA. Cells were pre-treated with 5 μg/ml Act-D, 2 h before the addition of 5 μM As^{3+}, 5 μM Cd^{2+}, 25 μM Cr^{6+} or 1 nM TCDD. Total RNA was collected 6 h after treatment using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to hybond-N nylon membranes, and hybridized with $\alpha^{32}P$-labeled Cyp1a1 and Gapdh cDNA probes. Graph represents Gapdh-standardized densitometric readings for Cyp1a1 mRNA expressed as a percent of Cyp1a1 mRNA levels in the presence of vehicle only (mean ± S.E.M., $n = 3$). One of three representative experiments is shown. $+p < 0.05$, compared to control; $*p < 0.05$, compared to respective Act-D treatment.



A)
As3+ (10 µM) - + - - - -
Cd2+ (25 µM) - - + - - -
Cr6+ (25 µM) - - - + - -
TCDD (20 nM) - - - - + +
100 X cold - - - - - +
AhR/XRE complex
B)
As3+ (10 µM) - + - - - -
Cd2+ (25 µM) - - + - - -
Cr6+ (25 µM) - - - + - -
TCDD (5 nM) - - - - + +
100 X cold - - - - - +
AhR/XRE complex
Non-specific binding

Figure 3.3. Effect of heavy metals on AhR-XRE binding. (A) In vitro AhR activity as measured by EMSA using guinea pig hepatic cytosolic extracts. (B) In vivo AhR activity as measured by EMSA using nuclear extracts prepared from Hepa 1c1c7 cells. Cytosolic and nuclear extracts were tested for binding activity to a γ^{32}P-labeled XRE consensus oligonucleotide. Binding reactions were carried out as described in Section 2, and the products were separated on a 4% polyacrylamide gel. The specificity of binding was confirmed by the competition of unlabeled XRE consensus oligonucleotide in 100-fold molar excess relative to the γ^{32}P-labeled probe, using extracts treated with TCDD. AhR-XRE complexes formed on the gel are indicated. One of three representative experiments is shown.

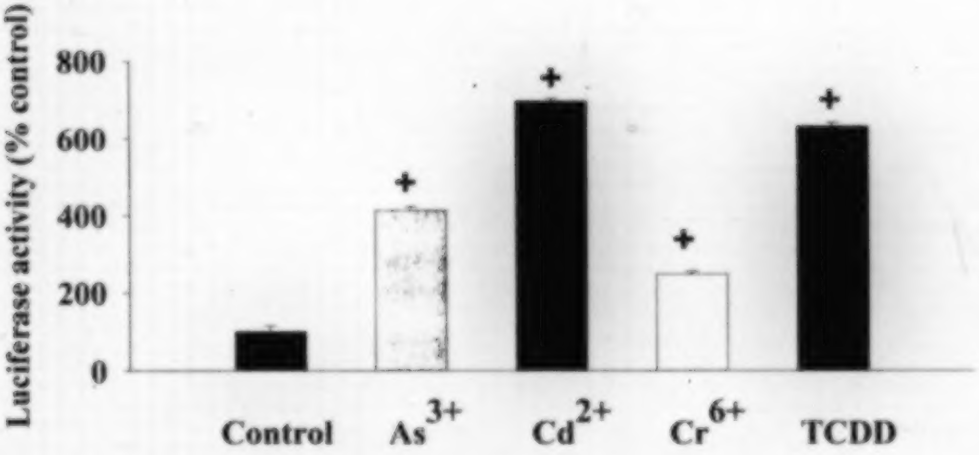


Figure 3.4. The effect of heavy metals on luciferase activity. Hepa 1c1c7 cells were transiently transfected with the XRE-luciferase transporter plasmid pGudLuc1. Cells were treated with vehicle, TCDD (5 nM), As^{3+} (10 μM), Cd^{2+} (25 μM), or Cr^{6+} (25 μM) for 24 h. Luciferase activity is reported as emitted light per well as a percent of control, vehicle treated cells (mean ± S.E.M., $n = 4$). $+p < 0.05$, compared to control.

3.3.4.0. Posttranscriptional modification of Cyp1a1 mRNA by heavy metals

The regulation of gene expression is multi-faceted and the level of mRNA expression is not only a function of the rate transcription, but is also dependent on the efficiency of its removal, through processing or degradation. Thus, it was vital to examine the effect of the heavy metals on the stability of Cyp1a1 mRNA transcripts. The Cyp1a1 mRNA half-life was assessed after nascent RNA transcription was stopped with Act-D. After an induction period of 12 h, the TCDD-induced Cyp1a1 mRNA decayed with a half-life of 4.69 ± 0.49 h (Figure 3.5). Interestingly, we observed that As^{3+} caused a marked and significant stabilization of the induced Cyp1a1 mRNA transcripts. Although Cd^{2+} and Cr^{6+} increased the Cyp1a1 mRNA half-life, it did not reach statistical significance (Figure 3.5). These results thus implicate the involvement of posttranscriptional mechanisms in the increase in the level of Cyp1a1 mRNA transcripts by As^{3+}.

3.3.5.0. Effect of protein synthesis and/or degradation on the regulation of Cyp1a1 mRNA by heavy metals

CHX has been shown to inhibit ligand-mediated degradation of the AhR, thus increasing the transcriptional activation of Cyp1a1 (Joiakim et al., 2004). Since AhR turnover can also be suppressed by the 26S proteasome inhibitor, MG-132 (Davarinos and Pollenz, 1999 and Ma and Baldwin, 2000), it was of interest to examine the effect of MG-132 on the induction of Cyp1a1 by the heavy metals in the absence and presence of CHX and MG-132.

Treatment of Hepa 1c1c7 cells with 1 μg/ml CHX or 10 μM MG-132 alone caused a 12- and 2-fold increase in the level of Cyp1a1 mRNA in vehicle-treated cells within 6 h of their addition (Figure 3.6; lanes 2 and 3, respectively). Not surprisingly, there was a further induction in the levels of Cyp1a1 mRNA with the addition of MG-132, 25 min before the addition of CHX. In cells treated with CHX and MG-132, the level of Cyp1a1 mRNA was 33-fold higher than the levels detected in vehicle-treated cells (Figure 3.6, lane 4). As expected, As^{3+} and Cd^{2+} significantly increased Cyp1a1 mRNA levels, compared to vehicle-treated cells (Figure 3.6, lanes 5, 9, and 13, respectively). Also, pre-

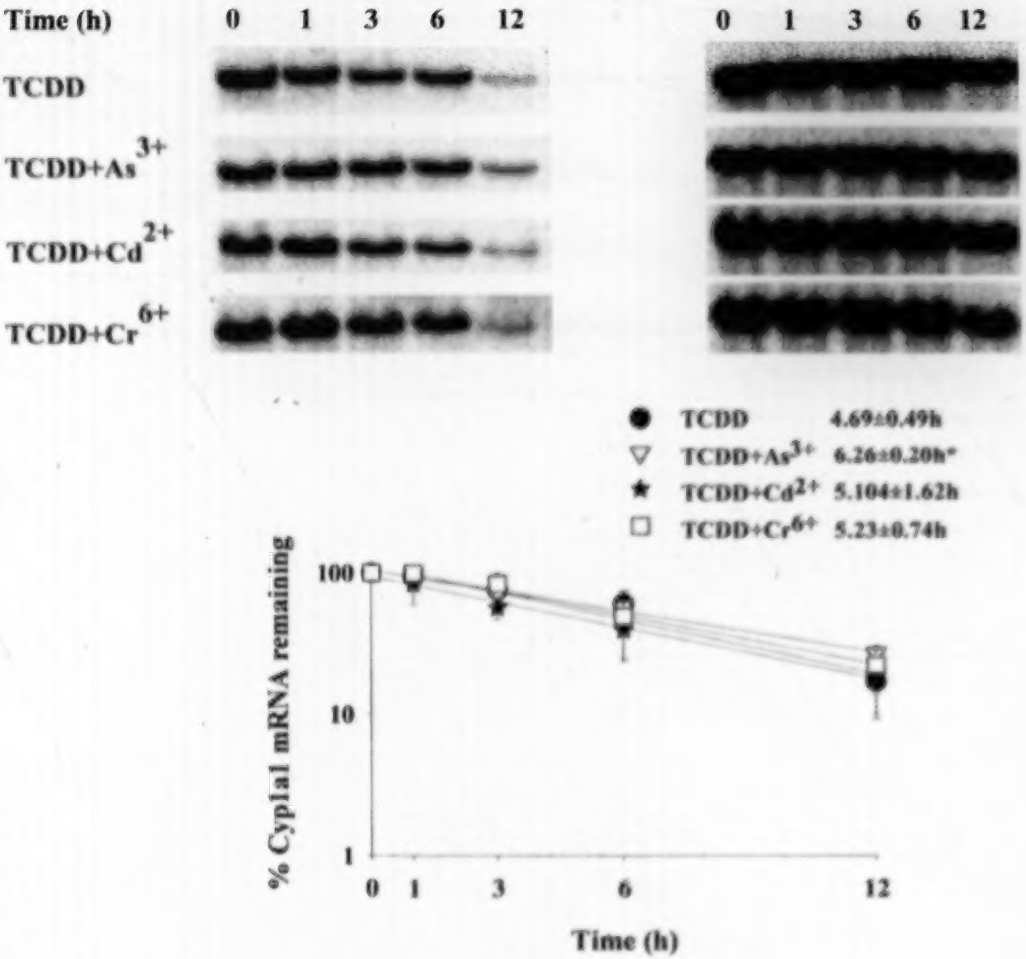


Figure 3.5. The effect of heavy metals on Cyp1a1 mRNA half-life. After cells were induced with 1 nM TCDD, 5 μg/ml of Act-D was added to inhibit further RNA synthesis, immediately before treatment with 5 μM As^{3+}, 5 μM Cd^{2+}, or 25 μM Cr^{6+}. Total RNA was extracted at 0, 1, 3, 6, and 12 h after incubation with the metals. Northern blot analysis was performed followed by hybridization with Cyp1a1 and Gapdh cDNA probes. The mRNA half-life values were determined from semilog plots of integrated densities versus time. *$p < 0.05$, compared to the degradation of TCDD-induced Cyp1a1 mRNA.

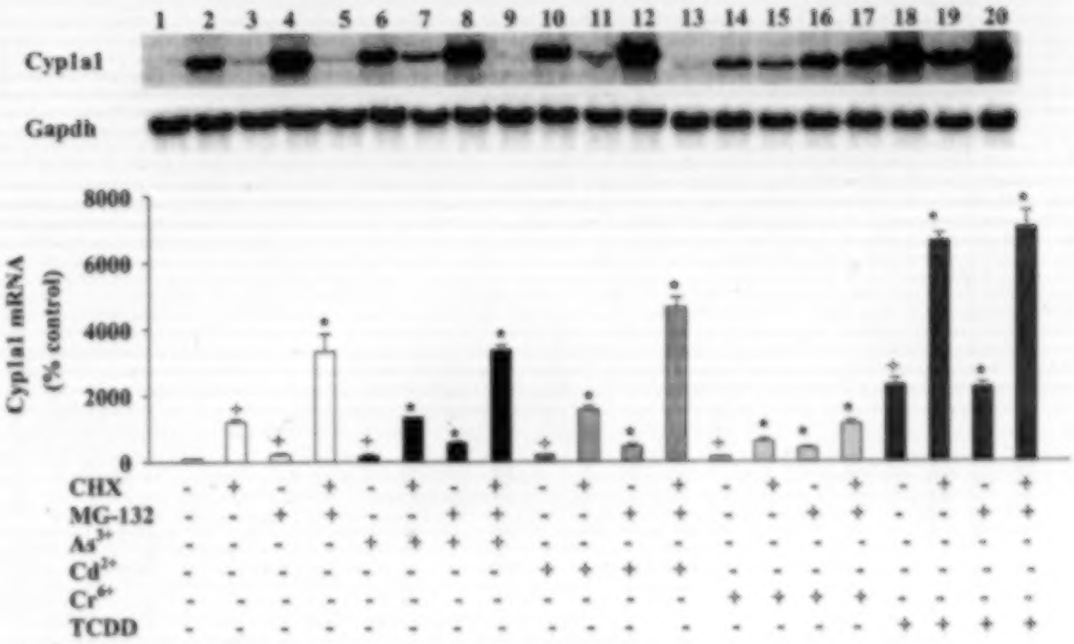


Figure 3.6. Effect of CHX and MG-132 on metal-mediated induction of Cyp1a1 mRNA. Cells were pre-treated with 10 μM MG-132 for 30 min and/or 1 μg/ml CHX for 5 min, before the addition of 5 μM As^{3+}, 5 μM Cd^{2+}, 25 μM Cr^{6+} or 1 nM TCDD. Total RNA was collected, 6 h after treatment with TCDD or the metals, using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to hybond-N nylon membranes, and hybridized with a α^{32}P-labeled Cyp1a1 and Gapdh cDNA probes. Graph represents Gapdh-standardized densitometric readings for Cyp1a1 mRNA expressed as a percent of Cyp1a1 mRNA levels in the presence of vehicle only (mean ± S.E.M., $n = 3$). One of three representative experiments is shown. $+p < 0.05$, compared to control; $*p < 0.05$, compared to respective CHX and/or MG-132 treatment.

treatment with CHX further potentiated the induction by As^{3+} and Cd^{2+} by five- and six-fold, respectively in a manner similar to the superinduction of TCDD-induced Cyp1a1 mRNA by CHX (Figure 3.6; lanes 6 and 10, respectively). Surprisingly, co-treatment of cells with MG-132 and As^{3+} or Cd^{2+} caused a further induction in the levels of Cyp1a1 mRNA by 2.8- and 2.2-fold, respectively, compared to MG-132 treated cells (Figure 3.6; lanes 7 and 11). As^{3+} and Cd^{2+} also further potentiated the increase in Cyp1a1 mRNA induced by the combination of CHX and MG-132 co-treatment, although the increase was only significant with Cd^{2+} treatment, which increased the induction of Cyp1a1 by 1.4-fold, compared to CHX and MG-132 treated cells (Figure 3.6; lanes 8 and 12). The effects of As^{3+} and Cd^{2+} on the regulation of Cyp1a1 mRNA closely resemble the pattern seen with the AhR ligand, TCDD. As can be seen in Figure 3.6, CHX further potentiated the TCDD-mediated induction of Cyp1a1 mRNA by three-fold (lane 18). Although MG-132 did not significantly alter the levels of TCDD-induced Cyp1a1 mRNA (Figure 3.7; lane 19), the combination of CHX and MG-132 further increased the TCDD-mediated induction of Cyp1a1 mRNA by 3.1 fold (Figure 3.6; lane 20).

Cr^{6+}, on the other hand, appears to display a differential effect on the regulation of Cyp1a1. Cr^{6+} alone significantly induced Cyp1a1 mRNA (Figure 3.6; lane 13). CHX superinduced the level of Cr^{6+}-induced Cyp1a1 mRNA by 4.2-fold (Figure 3.6; lane 14), but this level was 2-fold less than that obtained with treatment with CHX alone. Interestingly, there was a further induction in Cyp1a1 mRNA with MG-132 and Cr^{6+} co-treatment (Figure 3.6; lane 15). Also, the combination of CHX and MG-132 potentiated the Cr^{6+}-mediated Cyp1a1 mRNA induction by 7.8-fold but this level was 2.9-fold less than that obtained with CHX and MG-132 alone (Figure 3.6; lane 16).

Thus, it appears that *de novo* protein synthesis is not required for the induction of Cyp1a1 mRNA by any of the heavy metals. In fact, the superinduction of As^{3+}- and Cd^{2+}-mediated increase in Cyp1a1 mRNA suggests that TCDD, As^{3+} and Cd^{2+} share similar regulatory mechanisms in the regulation of *Cyp1a1* gene expression. The decrease in CHX-mediated Cyp1a1 mRNA accumulation by Cr^{6+} suggests a differential effect by Cr^{6+} on Cyp1a1 mRNA induction.

3.3.6.0. Modulation of Cyp1a1 activity by heavy metals

Cyp1a1 activity was determined in cells treated with various concentrations of TCDD (0 nM, 0.1 nM, 1 nM, and 10 nM) in the presence and absence of As^{3+}, Cd^{2+}, or Cr^{6+}. TCDD significantly induced Cyp1a1 activity at all concentrations tested, but in contrast to its effect on Cyp1a1 mRNA, the induction was not concentration-dependent (Figure 3.7). The most significant induction occurred with 1 nM TCDD which caused a 330-fold increase in Cyp1a1 activity. As^{3+}, Cd^{2+}, or Cr^{6+} alone did not alter Cyp1a1 activity in vehicle-treated cells (0 nM TCDD). However, all three metals inhibited TCDD-mediated induction of Cyp1a1 activity at all concentrations of TCDD. Of the three metals, Cr^{6+} appeared to be the most potent inhibitor at the concentration used. In fact, 25 μM of Cr^{6+} completely abolished Cyp1a1 activity induced by 0.1 nM, 1 nM, and 10 nM TCDD, bringing the levels of measured EROD activity back to the levels obtained in vehicle-treated cells (Figure 3.7). These results are contradictory to what is expected based on the effect of As^{3+} and Cd^{2+} on Cyp1a1 mRNA. Also, although we demonstrated the ability of Cr^{6+} to decrease TCDD-induced Cyp1a1 mRNA levels, its effect on Cyp1a1 activity are much more pronounced. Thus, posttranslational modifications must be responsible for the decrease in Cyp1a1 activity by As^{3+}, Cd^{2+}, and Cr^{6+}.

3.3.7.0. Effect of heavy metals on Cyp1a1 protein stability

In an attempt to examine whether or not posttranslational mechanisms are responsible for the decrease in Cyp1a1 activity by heavy metals, we first determined the effect of As^{3+}, Cd^{2+}, and Cr^{6+} on the stability of the Cyp1a1 protein. TCDD-induced Cyp1a1 protein degraded with a half-life of 8.16 ± 1.58 h (Figure 3.8). Surprisingly, Cd^{2+} and Cr^{6+} increased the stability of the Cyp1a1 protein (Figure 3.8). This effect was most pronounced with Cd^{2+}, which increased the Cyp1a1 protein half-life by 1.9-fold (Figure 3.8).

3.3.8.0. Effect of heavy metals on heme oxygenase-1 and total cellular heme content

To rationalize the ability of metals to increase Cyp1a1 protein half-life while decreasing its activity, we assessed the effect of the heavy metals on other posttranslational

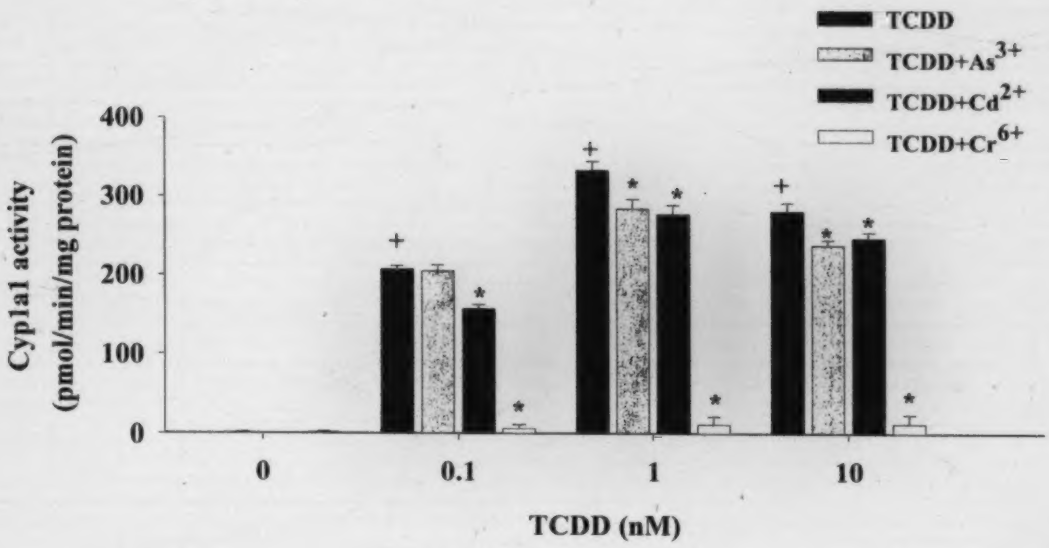


Figure 3.7. Effect of heavy metals on the concentration-dependent induction of Cyp1a1 activity. Cells were treated with 0 nM (vehicle), 0.1 nM, 1 nM, or 10 nM TCDD, in the presence and absence of 5 µM As^{3+}, 5 µM Cd^{2+}, or 25 µM Cr^{6+} for 24 h. Cyp1a1 activity was measured in intact living cells using 7-ethoxyresorufin as a substrate. Values are presented as mean ± S.E.M. ($n = 8$). $+p < 0.05$, compared to control; $*p < 0.05$, compared to respective TCDD treatment.

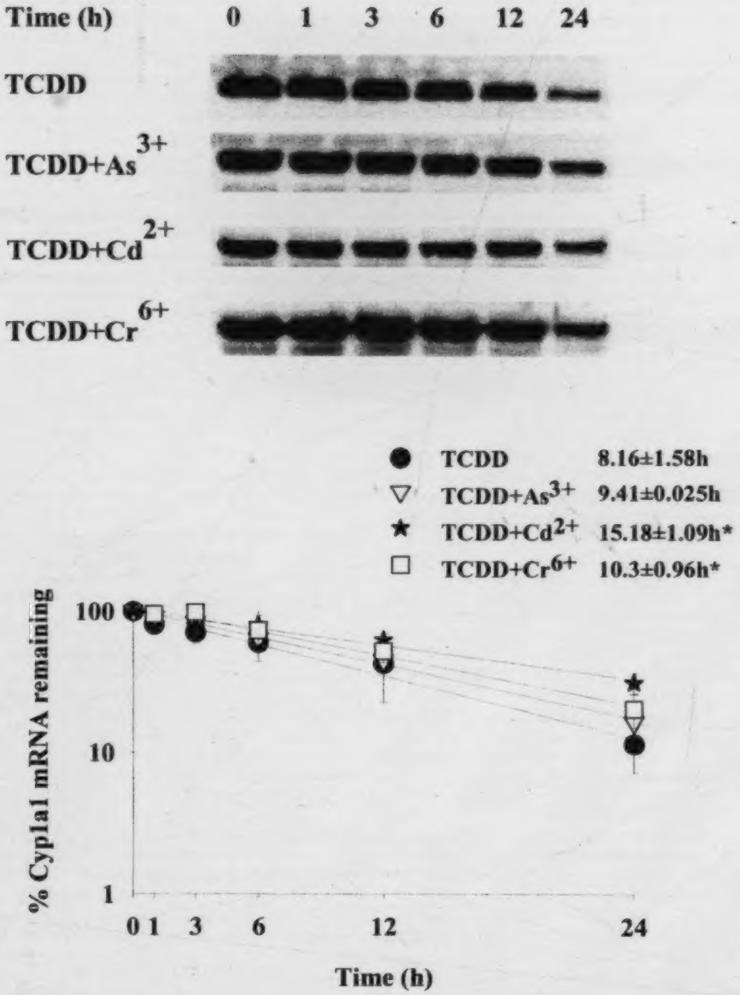


Figure 3.8. The effect of heavy metals on Cyp1a1 protein half-life. After cells were induced with 1 nM TCDD for 24 h, 10 μg/ml of CHX was added to inhibit further protein synthesis, immediately before treatment with 5 μM As^{3+}, 5 μM Cd^{2+}, or 25 μM Cr^{6+}. Protein was extracted at 0, 1, 3, 6, 12, and 24 h after incubation with the metals and subjected to Western blot analysis. The protein half-life values were determined from semilog plots of integrated densities vs. time. *$p < 0.05$, compared to the degradation of TCDD-induced Cyp1a1 protein.

mechanisms that may be involved in the modification of Cyp1a1 function. Like other members of the CYP superfamily, Cyp1a1 is a heme-containing protein, the absence of which results in loss of enzyme function. Hence, we measured the expression of the inducible HO-1 in Hepa 1c1c7 cells treated with various concentrations of TCDD in the presence and absence of As^{3+}, Cd^{2+}, or Cr^{6+}. TCDD alone did not alter HO-1 mRNA levels at all concentrations tested (0–10 nM) (Figure 3.9). However, when cells were co-treated with As^{3+}, there was a significant induction in HO-1 mRNA at all concentrations of TCDD (Figure 3.9). Interestingly, As^{3+} did not induce HO-1 mRNA to the same degree at all concentrations. The most potent induction occurred in cells treated with 0.1 nM TCDD and As^{3+}. Cd^{2+} and Cr^{6+} also significantly induced HO-1 mRNA at all concentrations of TCDD, although not to the same degree of induction observed with As^{3+} (Figure 3.9).

With the results of the effect of metals on HO-1 expression, it was imperative to assess how this induction impacts cellular heme content. Total cellular heme content was measured in Hepa 1c1c7 cells 24 h after treatment with 1 nM TCDD in the presence and absence of As^{3+}, Cd^{2+}, or Cr^{6+}. Surprisingly, TCDD significantly reduced the total cellular heme content at the concentration tested. In accordance with the HO-1 mRNA expression results, all three metals significantly decreased total cellular heme content compared to vehicle- and TCDD-treated cells (Figure 3.10). The potency of total cellular heme-content reduction however was dissimilar to their HO-1 mRNA inductive potency. As^{3+}, the most potent HO-1 mRNA inducer amongst the metals used, was the least potent inhibitor of total cellular cellular heme content. Cd^{2+} on the other hand reduced total cellular heme content to undetectable levels (Figure 3.10). The discrepancy noted in the difference of the effect of the metals in HO-1 mRNA and heme levels is likely due to varying abilities of the various metals to inhibit heme synthesis.

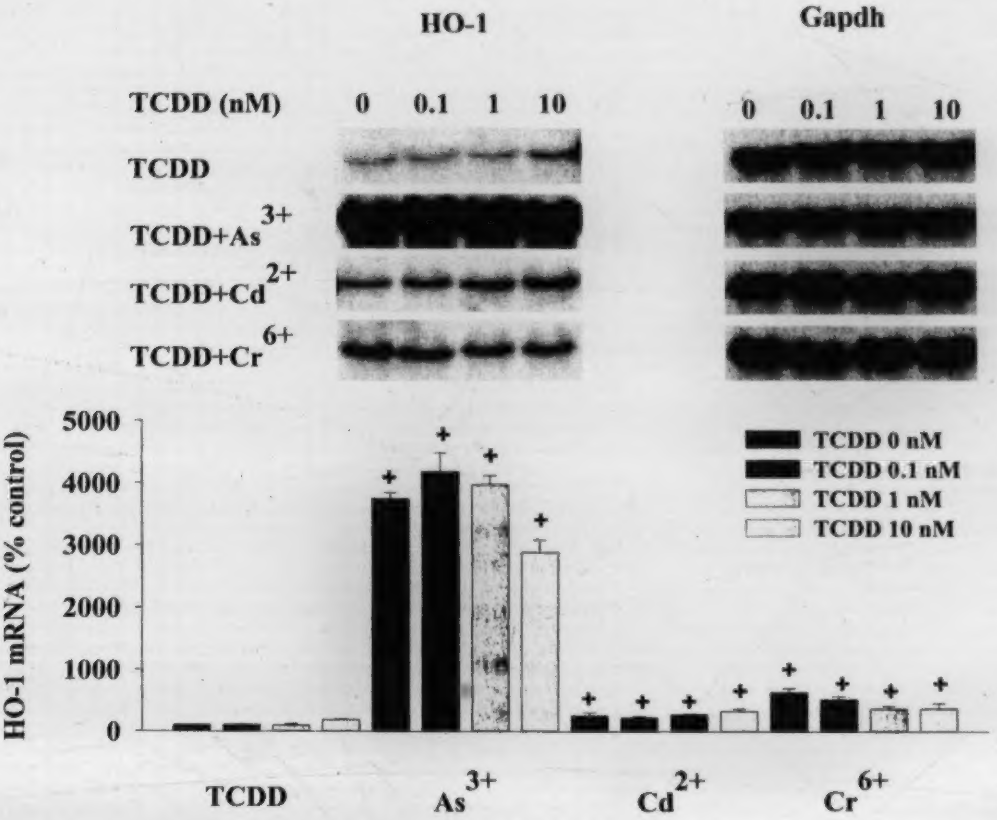


Figure 3.9. Effect of heavy metals on HO-1 mRNA. Cells were treated with 0 (vehicle), 0.1 nM, 1 nM, or 10 nM TCDD, in the presence and absence of 5 µM As^{3+}, 5 µM Cd^{2+}, or 25 µM Cr^{6+}. Total RNA was collected 6 h after treatment using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to hybond-N nylon membranes, and hybridized with a $\alpha^{32}P$-labeled HO-1 and Gapdh cDNA probes. Graphs represent Gapdh-standardized densitometric readings for HO-1 mRNA expressed as a percent of HO-1 mRNA levels in the presence of vehicle only (mean ± S.E.M., *n* = 3). One of three representative experiments is shown.

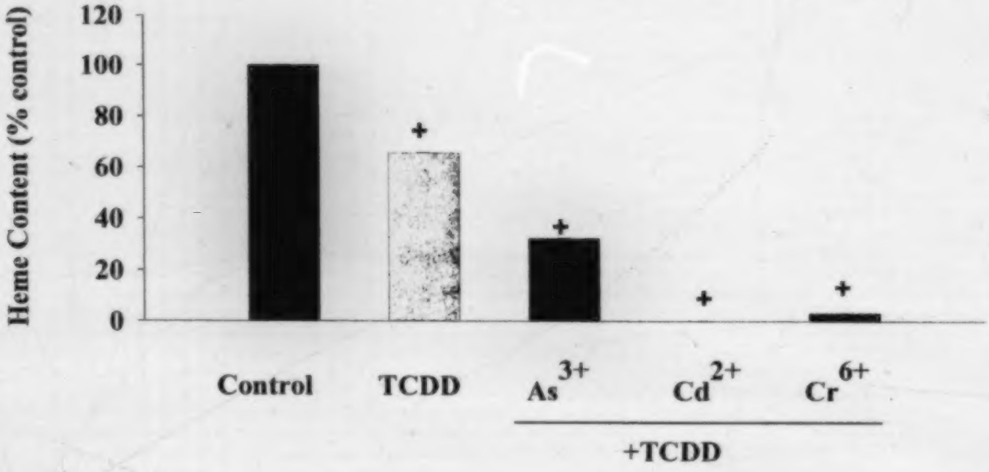


Figure 3.10. Effect of heavy metals on total cellular heme content. Cells were treated with 1 nM TCDD in the presence and absence of 5 μM As^{3+}, 5 μM Cd^{2+}, or 25 μM Cr^{6+}. After a 24 h incubation period, pelleted cells were boiled in oxalic acid, followed by resuspension in PBS. After centrifugation, the fluorescence of protoporphyrin IX was assayed by a spectrophotometric method. $+p < 0.05$, compared to control.

3.4.0.0. Discussion

Although the reduction of TCDD-induced Cyp1a1 activity by heavy metals has been clearly established, the molecular mechanisms through which such inhibition occurs remain controversial (Vakharia et al., 2001). In human HepG2 cells, transcriptional mechanisms have been attributed to the inhibition of CYP1A1 expression (Bessette et al., 2005). However, our previous studies indicate that the regulation of Cyp1a1 appears to be cell-specific (chapter 2).

In this study, to decipher the molecular mechanisms involved in the alteration of Cyp1a1 expression, we analyzed the effect of As^{3+}, Cd^{2+}, and Cr^{6+} on each step of the AhR-signaling pathway. Thus, we examined the effect of heavy metals on the transcriptional activation of Cyp1a1 and posttranscriptional and posttranslational modifications of the *Cyp1a1* gene products.

To closely examine the effect of the heavy metals on the expression of Cyp1a1 mRNA, we measured time-dependent changes in Cyp1a1 mRNA levels. As^{3+} and Cd^{2+} induced constitutive mRNA expression in a time-dependent manner, with maximal induction achieved at 3 h, and potentiated the time-dependent induction of Cyp1a1 mRNA up to 6 h after treatment. Cr^{6+}, on the other hand, caused a significant inhibition of the dose- and time-dependent induction of Cyp1a1 mRNA by TCDD, while causing a time-dependent induction in constitutive Cyp1a1 mRNA levels.

This evidence indicates that As^{3+}, Cd^{2+}, and Cr^{6+} act as inducers of Cyp1a1 by multiple mechanisms. In contrast to the response from TCDD, the constitutive induction and potentiation of TCDD-mediated response by all three metals was transient, dropping off within 12 h after treatment. The non-persistent effect of the metals is likely due to their cellular clearance by metabolic and/or oxidative mechanisms (Aposhian et al., 2004).

The cellular mRNA level, at any point in time, is a function of the rate of its production, by transcriptional mechanisms, and the rate of its degradation. From our results, it is apparent that As^{3+}, Cd^{2+} and Cr^{6+} induce mRNA expression by increasing the rate of transcription of the *Cyp1a1* gene and decreasing the degradation of the mRNA transcripts.

The ability of heavy metals to increase the transcription of Cyp1a1 is evidenced by the ability of the transcription inhibitor Act-D to abolish metal-induced Cyp1a1 mRNA. This induction in Cyp1a1 mRNA levels is independent of *de novo* protein synthesis since CHX did not inhibit the induction of Cyp1a1 mRNA by all three metals. In fact, CHX superinduced metal-induced Cyp1a1 mRNA. The results of the CHX experiment, however, shed light on the possible role of the AhR in the induction in *Cyp1a1* gene transcription by the heavy metals. Previous studies have already shown the ability of CHX to potentiate the induction of Cyp1a1 mRNA by TCDD (Ma et al., 2000). Since TCDD and other AhR ligands downregulate AhR levels through the ubiquitin-proteasome pathway (Roberts and Whitelaw, 1999, Santiago-Josefat et al., 2001), CHX is believed to superinduce TCDD-mediated induction of Cyp1a1 mRNA by inhibiting the synthesis of a labile protein, for the proteolysis of the AhR protein, thus increasing AhR stability and intensifying its effect on Cyp1a1 transcription (Ma et al., 2000). Since CHX also superinduces metal-mediated induction of Cyp1a1 mRNA in a similar manner, it is possible that metal-mediated induction of Cyp1a1 transcription is mediated by the AhR.

To further examine the role of the AhR in the transcriptional activation of Cyp1a1 by the heavy metals, we pre-treated the cells with the proteasome inhibitor MG-132 to prevent AhR degradation. In line with the results obtained when cells were pre-treated with CHX, MG-132 increased the level of Cyp1a1 mRNA induction by As^{3+}, Cd^{2+}, and Cr^{6+}. The level of induction was further potentiated when cells were pre-treated with MG-132 and CHX.

To confirm the ability of the metals to activate the AhR, EMSA experiments were performed using cytosolic and nuclear extracts. Cd^{2+} and Cr^{6+}, but not As^{3+}, induced nuclear accumulation of the AhR and AhR-DNA binding, albeit less efficiently than TCDD. Although nuclear accumulation of the AhR has been previously observed in Hepa 1c1c7 cells treated with As^{3+}, the mechanism responsible for this ligand-independent translocation was not addressed (Kann et al., 2005). The ability of the metals to activate AhR-dependent gene expression may suggest an interaction of the metals with the AhR. However, it is highly unlikely that metals act as AhR ligands themselves considering what is known about AhR-dependent mechanisms of gene activation (Denison and Nagy, 2003). Furthermore, using cytosolic extracts, we showed that neither As^{3+}, Cd^{2+}, nor Cr^{6+} induced the transformation and subsequent DNA binding of the AhR *in vitro*. Hence, it is apparent that the metals activate the AhR through an indirect mechanism. Heavy metals may alter some cellular metabolic pathways leading to the enhanced production of endogenous AhR ligands. The existence of endogenous AhR ligands has been suggested by numerous studies. The majority of these compounds however are weak inducers, which explains the relatively weak induction of Cyp1a1 mRNA when compared to TCDD. The first of these endogenous ligands are the heme degredation products bilirubin and biliverdin. Through the induction of HO-1, As^{3+}, Cd^{2+}, and Cr^{6+} may enhance the enzymatic conversion of heme to biliverdin, and subsequently, bilirubin (Albores et al., 1989). Experiments have not only demonstrated the ability of bilirubin to stimulate AhR transformation and DNA binding *in vitro* and in cell culture (Kapitulnik and Gonzalez, 1993), but it can also induce Cyp1a1 and a dioxin response element in a dose and AhR-dependent manner (Phelan et al., 1998, Sinal and Bend, 1997). Biliverdin has also been shown to activate XRE-dependent gene transcription, although it may be an indirect effect, by serving as a metabolic precursor to bilirubin (Denison and Nagy, 2003).

Another postulation implicates posttranscriptional modifications of histones in the increase in gene transcription. As^{3+} induces phosphorylation (He et al., 2003, Li et al., 2001) and acetylation (Li et al., 2001) of Histone H3 through the extracellular signal-regulated kinase (ERK), p38, and the Akt1 pathways. In accord, oxidative stress activates ERK (Aikawa et al., 1997), Akt (Ding et al., 2000), and p38 (Chuang et al., 2000). The phosphorylation-acetylation of histone H3 plays an important role in mediating transcriptional of many genes by increasing the association of RNA polymerase II with target genes (Li et al., 2001). Recently, histone deacetylation has been shown to be involved in the inducibility of Cyp1a1 mRNA transcription in human and mouse hepatoma cells (Shibazaki et al., 2004) and HeLa cells (Nakajima et al., 2003).

Thus, an increase in transcription and stabilization of mRNA are responsible for the detected increase in constitutive Cyp1a1 expression by As^{3+}, Cd^{2+}, and Cr^{6+} and the potentiation of TCDD-mediated induction of Cyp1a1 mRNA by As^{3+} and Cd^{2+}. The question of the mechanism by which Cr^{6+} inhibits inducible Cyp1a1 mRNA expression, however, is still left unanswered. Cr^{6+} significantly inhibited the dose- and time-dependent induction of Cyp1a1 mRNA by TCDD. Recently, Cr^{6+} was shown to inhibit benzo[a]pyrene-mediated release of HDAC-1 from chromatin, preventing the recruitment of the co-activator p300 to the Cyp1a1 transcriptional domain, hence blocking inducible gene transcription (Wei et al., 2004).

At this point, we offer no explanation for the stabilization of Cyp1a1 mRNA by the heavy metals. mRNA degradation is a regulated process, since the half-lives of some mRNAs are known to change throughout the cell cycle, and can be changed in response to extracellular signals (Wilusz et al., 2001). We speculate that heavy metals may act by several mechanisms to increase the stability of mRNA.

Despite the increase in the Cyp1a1 mRNA levels, As^{3+}, Cd^{2+}, and Cr^{6+} cause a significant inhibition in the TCDD-induced Cyp1a1 activity. Surprisingly, this decrease in activity is not mediated by a reduction in Cyp1a1 protein since the heavy metals increased the stability of induced protein levels. Little is known about the mechanisms involved in Cyp1a1 protein degradation, but we do know that these mechanisms do not appear to involve the 26S proteasome (Roberts and Whitelaw, 1999). PI3K and PKC may have a role in protein stabilization (Zhou et al., 2004). It is still not known whether these signaling pathways play a role in stabilizing Cyp1a1, though it has already been reported that As^{3+} activates PI3K/Akt signaling through the generation of reactive oxygen species (ROS) (Gao et al., 2004). Metals may also stabilize the Cyp1a1 protein by direct binding to the apoprotein, rendering it less susceptible to proteases.

In spite of the increase in Cyp1a1 protein stability, heavy metals decrease Cyp1a1 activity, likely by multiple mechanisms. One mechanism is the induction of HO-1, since all three metals cause a significant induction in HO-1 mRNA expression and decrease in cellular heme content. However, the induction of HO-1 does not explain why Cd^{2+}, the most potent inhibitor of heme content, did not cause the most significant reduction in Cyp1a1 activity. Furthermore, the addition of increasing concentrations of heme, which is also a potent inducer of HO-1, alters CYP450 expression while co-treatment of cells with As^{3+} and heme diminished CYP2B1/2 and CYP1A1 activities in rat hepatocyte cultures (Jacobs et al., 1999). Hence, although HO-1 induction may contribute to the inhibition of Cyp1a1 activity by the metals, there must other interplaying mechanisms.

In conclusion, the present study demonstrates that As^{3+}, Cd^{2+}, and Cr^{6+} cause activation of the AhR and transcriptional induction of Cyp1a1. The three metals are also involved in posttranscriptional modifications, causing an increase in the stability of Cyp1a1 mRNA transcripts, while decreasing the degradation of the Cyp1a1 protein and inhibiting its activity.

3.5.0.0. References

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4.0.0.0. Chapter 4.0

ARSENITE AND CADMIUM, BUT NOT CHROMIUM, INDUCE NAD(P)H:QUINONE OXIDOREDUCTASE 1 THROUGH TRANSCRIPTIONAL MECHANISMS, IN SPITE OF POST-TRANSCRIPTIONAL MODIFICATIONS.

A version of this chapter has been submitted: Elbekai, R.H. and El-Kadi, A.O., 2007.

4.1.0.0. Introduction

Aromatic compounds with oxygen-containing substituents can be converted enzymatically and non-enzymatically to cytotoxic, quinone-containing byproducts. Quinones exert their toxic effects by multiple mechanisms, including the generation of reactive oxygen species and their conversion to DNA-binding semiquinone free radicals. Arylating quinones also react with cellular thiols, forming quinone–thiol adducts (Monks and Jones, 2002). Polycyclic aromatic hydrocarbons (PAHs) represent a class of compounds whose toxicity may be attributed to the formation of quinone-containing metabolites. PAHs are ubiquitous and persistent environmental contaminants, often released into the air, soil, and water from natural and anthropogenic sources. Forest fires and volcano emissions are examples of natural sources of airborne PAHs (Baek et al., 1991). On the other hand, the production of coal tar (Baek et al., 1991), industrial power generation (Dor et al., 1999), wood treatment plants, and automotive exhaust (Baek et al., 1991) serve as major anthropogenic sources.

Interestingly, PAHs induce a host of biological responses, including the induction of enzymes responsible for their metabolism, through binding to and activating the aryl hydrocarbon receptor (AhR). Activation of the AhR results in transcriptional activation and the up-regulation of Phase I and Phase II drug metabolizing enzymes (Swanson, 2002).

Metabolic activation of PAHs into their quinone-containing metabolites is mediated primarily by members of the phase I drug metabolizing enzymes, specifically Cyp1a1 (Gelboin, 1980). The induction of the phase II enzymes, such as Nqo1, serves as an adaptive mechanism to decrease the deleterious effect of these mutagenic metabolites.

Nqo1 is a homodimeric flavoprotein that catalyses the detoxication of quinones through a single step, two-electron reduction process (Nioi and Hayes, 2004, Talalay and Dinkova-Kostova, 2004). In addition to detoxifying quinones, Nqo1 also helps to maintain endogenous antioxidants, specifically ubiquinone and α-tocopherol-quinone, in their reduced forms (Landi et al., 1997, Siegel et al., 1997). Thus, the oxidoreductase plays an important cytoprotective role.

Consequently, Chemicals capable of inducing Nqo1 and other detoxifying enzymes, or inhibiting Cyp450, may block the mutagenicity and carcinogenicity of these environmental pollutants (Zhang et al., 1992, Zhang et al., 1994). Interestingly, AhR ligands co-exist in the environment with heavy metals, typified by arsenite (As^{3+}), cadmium (Cd^{2+}), and chromium (Cr^{6+}). In our previous studies, we demonstrated the ability of these heavy metals to modulate the induction of Nqo1 by various AhR ligands. However, the molecular mechanisms mediating the interaction between AhR ligands and heavy metals remain to be discovered.

Widespread studies have unveiled the presence of not only the XRE, but another regulatory element in the 5′ flanking region of the *Nqo1* gene: the antioxidant response element (ARE). The ARE mediates the basal expression as well as induction of Nqo1 in response to reactive oxygen species, antioxidants, and tumor promoters (Jaiswal, 1994, Li and Jaiswal, 1994). In fact, regulation of Nqo1 through the ARE is part of a cellular defense mechanism responsible for the induction of other enzymes in response to oxidative stress (Jaiswal, 1994, Rushmore and Pickett, 1993). Such genes include UDP-glucuronosyl transferases (UDP-GT) and enzymes involved in glutathione homeostasis, such as glutathione S-transferases (GSTs) and γ-glutamylcysteine synthetase (γ-GCS) (Mulcahy et al., 1997).

Although the transcriptional regulation of Nqo1 is well documented, very little information about possible posttranscriptional regulation of the Nqo1 gene products, and the susceptibility of Nqo1 to modifiers of its activity, is available. In an attempt to identify the molecular targets and pathways susceptible to modification by the heavy metals, we examined the effect of As^{3+}, Cd^{2+}, and Cr^{6+} on Nqo1 expression at the various pathways required for full expression of Nqo1 protein activity. Hepa 1c1c7 cells were used as they are a highly responsive cell line to AhR ligands. Northern blot analysis was used to construct the time course of Nqo1 mRNA. To assess the effect of the metals on transcriptional regulation, mRNA levels were assessed in the presence of the actinomycin

D (Act-D) or cycloheximide (CHX). Studies with Act-D and CHX were also carried out to determine the effect of the metals on Nqo1 mRNA and protein stability, respectively. Here, we report for the first time transcriptional and posttranscriptional modification of Nqo1 by heavy metals.

4.2.0.0. Materials and Methods

4.2.1.0. Materials.

Sodium arsenite, cadmium chloride, chromium trioxide, cycloheximide, anti-rabbit IgG peroxidase secondary antibody, and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-tetrachlorodibenzo-p-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent, and the random primers DNA labeling system were purchased from Invitrogen Co. (Grand Island, NY). Actinomycin D and MG-132 were purchased from Calbiochem (San Diego, CA). Chemiluminescence western blotting detection reagents and hybond-N-nylon membranes were from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). polyclonal primary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [α-^{32}P]dCTP was supplied by the DNA Core Services Laboratory, University of Alberta. All other chemicals were purchased from Fisher Scientific (Toronto, ON).

4.2.2.0. Cell culture.

Hepa 1c1c7 cell lines, generously provided by Dr. O. Hankinson at the University of California (Los Angeles, CA), were maintained in Dulbecco's Modified Eagle's Medium (DMEM), without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamycin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37°C in a 5% CO_2 humidified incubator.

4.2.3.0. Chemical treatments.

Cells were treated in serum free medium with TCDD, As^{3+}, Cd^{2+}, or Cr^{6+}, actinomycin D (Act-D) or cycloheximide (CHX), as described in figure legends. DMSO was used as the solvent for TCDD and MG-132. The metals and CHX were dissolved in double de-ionized water. Act-D was dissolved in 95% ethanol. TCDD and MG-132 were maintained in DMSO at -20°C until use. All other reagents were prepared freshly for each treatment. In all treatments, the concentration of double de-ionized water in treatment medium did not exceed 0.5% (v/v) while the DMSO concentration did not exceed 0.05% (v/v).

4.2.4.0. RNA extraction and analysis

After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen Co). Northern blot analysis of total RNA was performed as described elsewhere (chapter 2). The cDNA probes for mouse Nqo1 and Gapdh mRNA were generously provided by Dr. John R. Bend (University of Western Ontario, London, ON). All probes were [α-^{32}P]-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions.

4.2.5.0. mRNA stability

The half-life of Nqo1 mRNA was analyzed by an Act-D-chase assay. Cells were pre-treated with 1 nM TCDD for 12 h. Cells were then washed and incubated with 5 μg/ml Act-D, to block further RNA synthesis, immediately before treatment with 5 μM As^{3+} or Cd^{2+}, or 25 μM Cr^{6+}. Total RNA was extracted from metal-exposed cells 0, 1, 3, 6, 12, and 24 h after treatment. Northern blot analysis was performed followed by hybridization first with the Nqo1 probe and then with the Gapdh probe. Band densities were quantified using the ImageJ image processing program (National Institute of Mental Health, Bethesda, MD). The mRNA half-life values were determined from semilog plots of integrated densities versus time.

4.2.6.0. Protein stability

The half-life of Nqo1 protein was analyzed by CHX-chase assay. Cells were pre-treated with 1 nM TCDD for 24 h. Cells were then washed and incubated with 10 μg/ml CHX, to inhibit further protein synthesis, immediately before treatment with 5 μM As^{3+} or Cd^{2+}, or 25 μM Cr^{6+}. Cells were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 μl/ml of protease inhibitor cocktail, at 0, 1, 3, 6, 12, and 24 h after incubation with the metals. The cellular homogenatess were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Proteins (25 μg) were separated by 10% SDS-PAGE bis-acrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane as described previously (chapter 2). After blocking overnight in blocking buffer, protein blots were incubated with a primary polyclonal rabbit anti-mouse Nqo1 antibody for 18 h at 4°C. This was followed by incubation with a peroxidase-conjugated rabbit anti-mouse IgG secondary antibody, in blocking buffer, for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (GE Healthcare Life Sciences, Piscataway, NJ). Band densities were also then quantified using the ImageJ image processing program and the protein half-life values were determined from semilog plots of integrated densities versus time.

4.2.7.0. Determination of Nqo1 activity

Cells were grown for 48 h in 6 well plates before treatment with TCDD and/or metals. After the elapsed treatment period, cells were washed with PBS and kept in 0.5ml of homogenization buffer, in a -80°C freezer, for at least 24 h. Thawed cells were extracted and homogenized with a Kontes homogenizer and then centrifuged at 10 000 x *g* for 20 min. The supernatant fractions were then removed and protein concentrations in the supernatant were determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as the standard. Nqo1 activity was determined by the continuous spectrophotometric assay of Ernster (Ernster, 1967), which quantitates the reduction of

2,6-dichlorophenolindophenol (DCPIP) by 0.01 mg of cell homogenate protein in the presence of β-nicotinamide adenine dinucleotide phosphate (NADPH; 200 μM) and flavin adenine dinucleotide (FAD; 5 μM). The rate of reduction of DCPIP (40 μM) in 1 ml of Tris-HCl buffer (pH 7.8, 25 mM) containing 0.1% (v/v) Tween 20 and 0.023% bovine serum albumin was monitored for 90 sec at 600 nm with $\varepsilon = 2.1\ \text{mM}^{-1}\ \text{cm}^{-1}$.

4.2.8.0. Statistical analysis

All results are presented as mean ± S.E.M. The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc comparison. The differences were considered significant when $P<0.05$.

4.3.0.0. Results

4.3.1.0. Effect of heavy metals on the time-dependent induction of Nqo1 mRNA

We analyzed the time course of *Nqo1* gene expression at various time points up to 24 h after treatment, at first, with the metals alone. All three metals provoked a time-dependent induction of Nqo1 mRNA, albeit in a dissimilar manner (Figure 4.1A). An increase in Nqo1 mRNA was apparent as early as 1 h after treatment of Hepa 1c1c7 cells with As^{3+} and Cr^{6+}, whereas up to 3 h was needed for a significant increase in Nqo1 mRNA to be apparent after treatment with Cd^{2+}. With all three metals however, induction was maximal within 6 h of treatment. As^{3+}, the most potent Nqo1 mRNA inducer among the metals, caused a maximal 3.4-fold increase in Nqo1 mRNA 6 h after treatment (Figure 4.1A). Interestingly, metal-induced mRNA transcripts were rapidly degraded as there was an apparent significant decrease in Nqo1 mRNA levels 24 h after treatment of cells with As^{3+}, Cd^{2+}, and Cr^{6+} (Figure 4.1A). Gapdh mRNA, which was used as a loading control, remained stable over the time period of the study.

In a similar manner, we assessed the effect of each of the heavy metals on the time course of Nqo1 mRNA induction in the presence of a single concentration of TCDD (1 nM). When Hepa 1c1c7 cells were treated with TCDD alone, time course analysis revealed

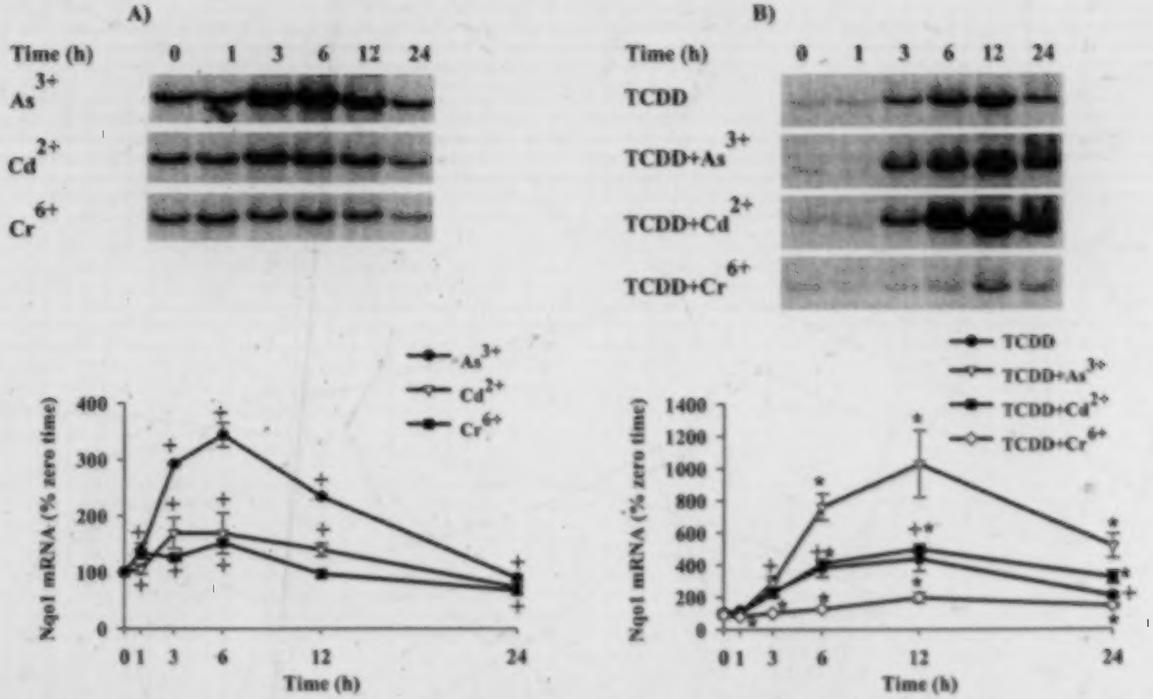

Figure 4.1. Time-dependent effects of heavy metals on the constitutive Nqo1 (A) and Inducible (B) Nqo1 mRNA levels. Cells were treated with 5 µM As^{3+}, Cd^{2+}, or 25 µM Cr^{6+} in the presence or absence of 1 nM TCDD for the time point indicated. Total cellular RNA was collected at 0, 1, 3, 6, 12, and 24 h after treatment using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to hybond-N nylon membranes, and hybridized with [α^{32}P]-labeled Cyp1a1 and Gapdh cDNA probes. Graph represents Gapdh-standardized densitometric readings for Nqo1 mRNA expressed as a percent of mRNA levels in the presence of vehicle only (mean ± SEM, $n = 3$). One of three representative experiments is shown. + $p<0.05$, compared to control; * $p<0.05$, compared to respective TCDD treatment.

that induction was maximal by 12 h but was maintained up to 24 h after treatment (Figure 4.1B). Addition of As^{3+} significantly potentiated the induction of Nqo1 mRNA at all measured time points, amounting to a maximal 2.5-fold increase at 24 h post-treatment (Figure 4.1B). Similarly, Cd^{2+} potentiated the induction of Nqo1 mRNA by TCDD, although this potentiation was somewhat delayed, occurring 6-24 h after treatment (Figure 4.1B). Consistent with its effects on inducible mRNA levels, Cr^{6+} significantly inhibited the TCDD-mediated time-dependent induction of Nqo1 mRNA at all measured time points (Figure 4.1B).

4.3.2.0. Effect of heavy metals on concentration-dependent induction of Nqo1 mRNA by TCDD

To determine the presence of a concentration-dependent component in the modulation of *Nqo1* gene expression, we determined the effect of As^{3+}, Cd^{2+}, and Cr^{6+} on the TCDD-mediated induction of Nqo1 mRNA using various concentrations of TCDD ranging from 0 to 10 nM. In Hepa 1c1c7 cells, a 6 h incubation period with TCDD produced a concentration-dependent increase in Nqo1 mRNA (Figure 4.2). Induction was statistically significant across the entire range of TCDD concentrations tested, with maximal increase occurring at the highest concentration tested (10 nM), which produced a 6.3-fold increase in Nqo1 mRNA. Interestingly, the induction of Nqo1 mRNA was not saturable. As^{3+} further potentiated the induction of Nqo1 mRNA with all concentrations of TCDD, although the potentiation was particularly apparent at the lower end of the TCDD concentration range (Figure 4.2). Cd^{2+} also potentiated the induction of Nqo1 with all TCDD concentrations. However, the fold increase was consistent across all concentrations of TCDD, indicating an additive, not synergistic effect. On the other hand, Cr^{6+} inhibited Nqo1-induced mRNA levels by 2.7-, 3.9-, 5.4-, and 6.7-fold with 0, 0.1, 1, and 10 nM TCDD.

4.3.3.0. Transcriptional effects by heavy metals

To examine the role of transcriptional mechanisms in the modulation of Nqo1 mRNA by

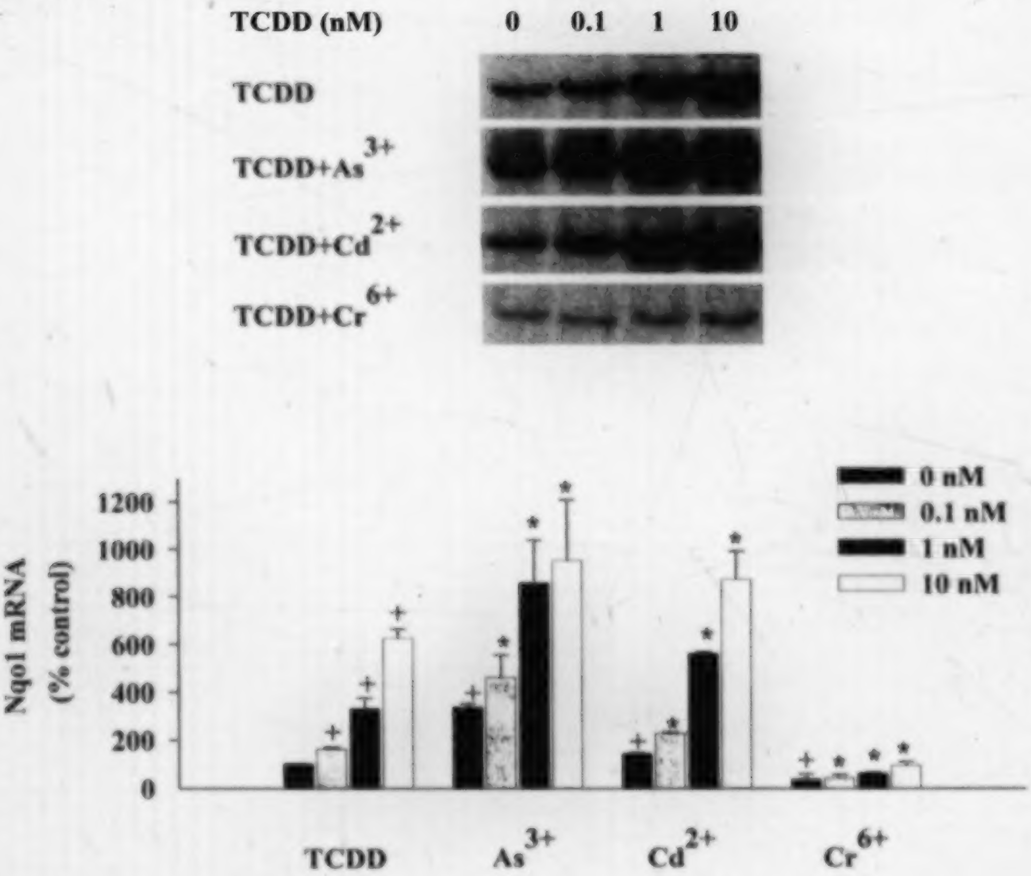

Figure 4.2. Effect of heavy metals on the concentration-dependent induction of Nqo1 mRNA. Cells were treated with 0 (vehicle), 0.1, 1, or 10 nM TCDD in the presence and absence of 5 μM As^{3+}, Cd^{2+}, or 25 μM Cr^{6+}. Total cellular RNA was collected using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to hybond-N nylon membranes, and hybridized with [$\alpha^{32}P$]-labeled Nqo1 and Gapdh cDNA probes. Graph represents Gapdh-standardized densitometric readings for Nqo1 mRNA expressed as a percent of mRNA levels in the presence of vehicle only (mean ± SEM, $n = 3$). One of three representative experiments is shown. + $p<0.05$, compared to control; * $p<0.05$, compared to respective TCDD treatment.

heavy metals, a transcription inhibitor was used to prevent *de novo* RNA synthesis. Act-D, a blocker of DNA-dependent RNA synthesis, reduced constitutive Nqo1 mRNA levels by 2-fold (Figure 4.3A). As expected, TCDD induced Nqo1 mRNA by 1.7 fold (Figure 4.3A). This induction, however, was completely blocked in the presence of Act-D, verifying the role of transcriptional mechanisms in the induction of Nqo1 by TCDD. While the heavy metals had differential effects on Nqo1 mRNA levels, the presence of Act-D was consistently associated with a reduction in Nqo1 mRNA levels. As^{3+} and Cd^{2+} increased Nqo1 mRNA levels by 1.9- and 1.4- fold, respectively; however, there was no statistically significant difference in Nqo1 mRNA levels from cells treated with Act-D alone and those from cells treated with As^{3+} or Cd^{2+} with Act-D (Figure 4.3A).

Interestingly, although Cr^{6+} decreased Nqo1 mRNA at the measured time point, the presence of Act-D caused a further decrease in Nqo1 mRNA levels (Figure 4.3A).

4.3.4.0. Basal and metal-inducible Nqo1 requires a labile factor

Since a labile and/or inducible protein is required for the constitutive and TCDD-inducible Nqo1 expression (Ma et al., 2004), we sought to determine whether the heavy metals induce Nqo1 by a similar mechanism. To do this, Hepa 1c1c7 cells were treated with 1 μg/mL of the protein synthesis inhibitor CHX, 5 min before treatment with As^{3+}, Cd^{2+}, Cr^{6+} or TCDD. In agreement with other previously published results, CHX inhibited the constitutive expression of Nqo1 mRNA, by more than 60% (Figure 4.3B). While TCDD induced Nqo1 mRNA, the presence of CHX also reduced the levels of Nqo1 mRNA to those of CHX alone. In a similar manner, CHX inhibited the As^{3+}-mediated induction of Nqo1 mRNA, albeit, the levels in this case being higher than those obtained with CHX alone (Figure 4.3B). CHX also decreased Nqo1 mRNA levels in the presence of Cd^{2+} and Cr^{6+} to levels below, or similar to those obtained with CHX alone (Figure 4.3B). Thus, similar to TCDD, heavy metal-mediated induction of Nqo1 requires de novo protein synthesis.

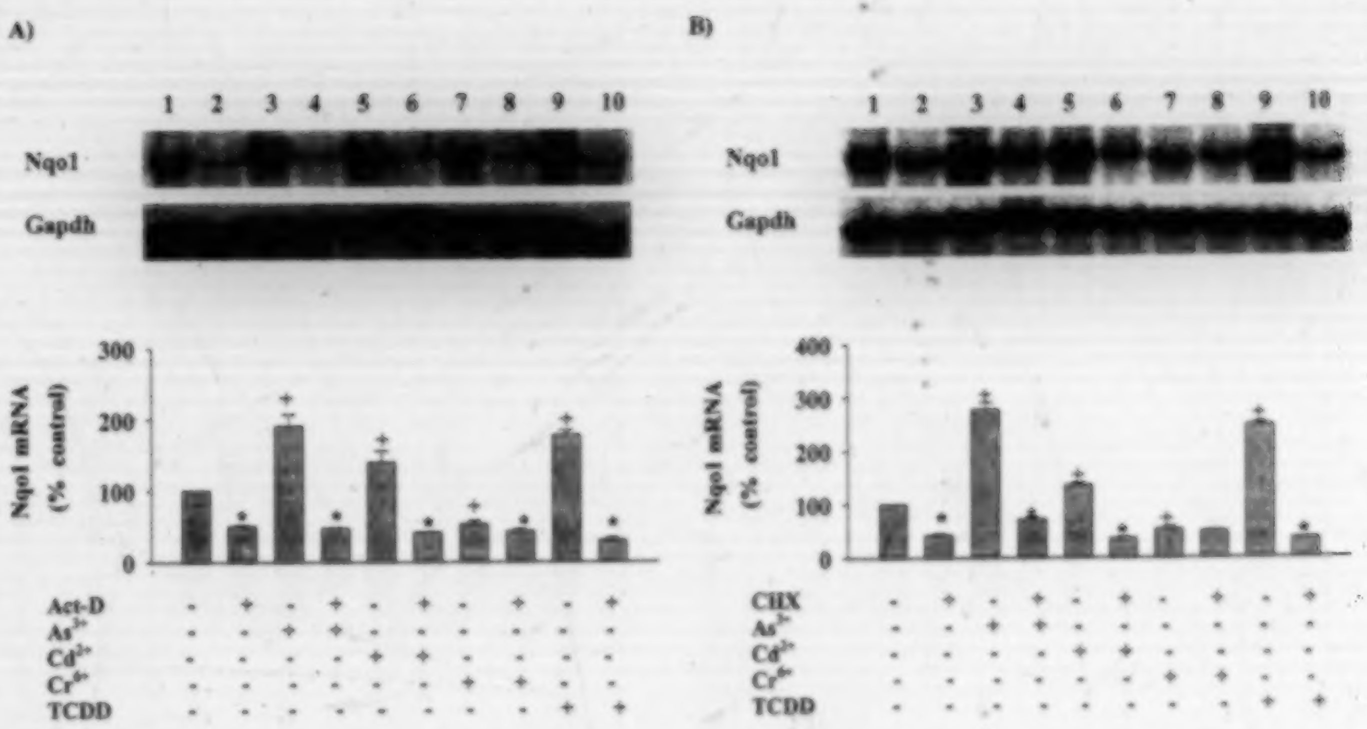

Figure 4.3. Effect of Act-D (A) and CHX (B) on metal-mediated induction of Nqo1 mRNA. Cells were pre-treated with 5 µg/mL Act-D, 2 h before, or 1 µg/mL CHX, 5 min before the addition of 5 µM As^{3+}, Cd^{2+}, 25 µM Cr^{6+} or 1nM TCDD. Total RNA was collected, 6 h after treatment using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to hybond-N nylon membranes, and hybridized with [$\alpha^{32}P$]-labeled Nqo1 and Gapdh cDNA probes. Graph represents Gapdh-standardized densitometric readings for Nqo1 mRNA expressed as a percent of mRNA levels in the presence of vehicle only (mean ± SEM, $n = 3$). One of three representative experiments is shown. + $p<0.05$, compared to control; * $p<0.05$, compared to respective Act-D or CHX treatment.

4.3.5.0. Effect of heavy metals on Nqo1 mRNA turnover

In order to test whether changes in mRNA stability contribute to the changes in mRNA steady-state levels observed, we followed the decay of transcripts encoding Nqo1 at 0-24 h after pharmacological arrest of transcription by Act-D. All half-lives we reported were determined from goodness-of-fit criterion using log-linear graphs with regression coefficients greater than 0.8. Half-life for the TCDD-induced Nqo1 mRNA was calculated to be 13.17 h (Figure 4.4). Surprisingly, all three metals significantly decreased Nqo1 mRNA half-life. The calculated Nqo1 mRNA half-life was 7.38, 8.2, and 5.86 h in the presence of As^{3+}, Cd^{2+}, and Cr^{6+}, respectively (Figure 4.4). This is to our knowledge the first report of a selective posttranscriptional effect by heavy metals on Nqo1 mRNA transcripts.

4.3.6.0. Effect of heavy metals on Nqo1 protein turnover

With the data of the mRNA stability in light, it was of interest to examine the effect of the heavy metals on the turnover rate of the final gene product, the Nqo1 protein. In an attempt to examine the involvement of posttranslational mechanisms in the modulation of Nqo1 activity by heavy metals, we determined the effect of As^{3+}, Cd^{2+}, and Cr^{6+} on the stability of the Nqo1 protein. After a 24 h induction period with TCDD, cycloheximide was added to halt protein synthesis and protein was harvested at 0-24 h in the presence and absence of each of the metals. Western blot analysis revealed a fairly long protein half-life, lasting longer than 24 h and none of the metals appeared to alter protein stability (Figure 4.5).

4.3.7.0. Effect of heavy metals on Nqo1 activity

Our investigations thus far have implicated transcriptional, posttranscriptional, and even posttranslational modulation of the phase II gene products by the heavy metals. In an attempt to provide a functional link between these early effect and more downstream events, we measured the activity in Nqo1 using various doses of TCDD (0-10 nM)) in the presence and absence of As^{3+}, Cd^{2}, or Cr^{6+}. TCDD induced Nqo1 activity at all

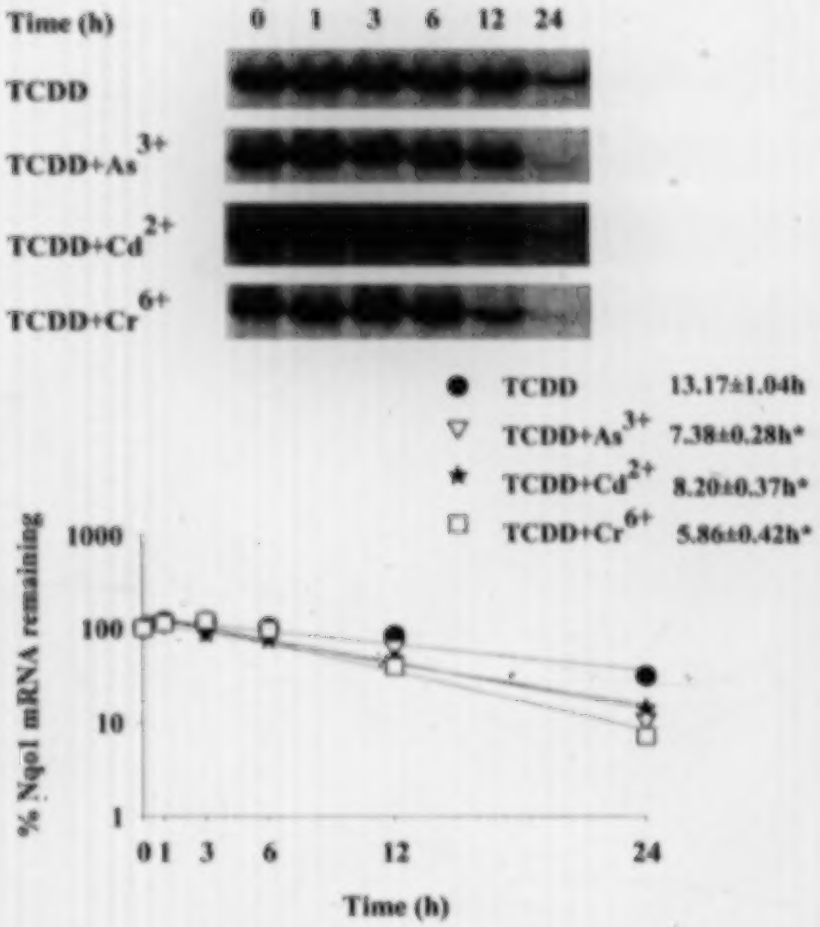

Figure 4.4. The effect of heavy metals on Nqo1 mRNA half-life. After cells were induced with 1 nM TCDD, 5 µg/ml of Act-D was added to inhibit further RNA synthesis, immediately before treatment with 5 µM As^{3+}, Cd^{2+}, or 25 µM Cr^{6+}. Total RNA was extracted at 0, 1, 3, 6, 12, and 24 h after incubation with the metals. Northern blot analysis was performed followed by hybridization with Nqo1 and Gapdh cDNA probes. The mRNA half-life values were determined from semilog plots of integrated densities versus time. * $p<0.05$, compared to the degradation of TCDD-induced Nqo1 mRNA.

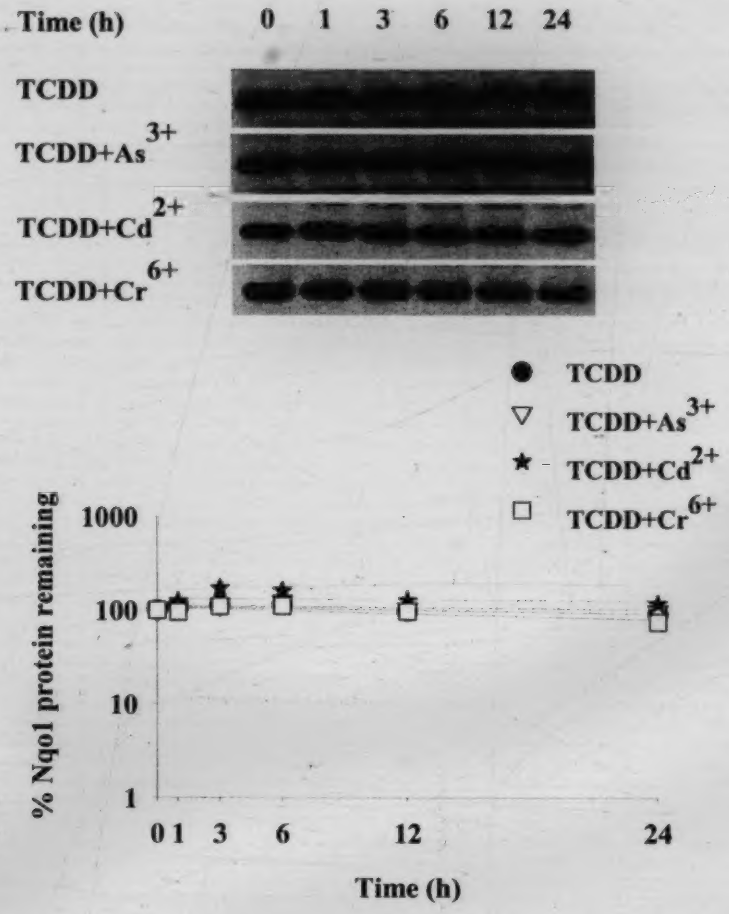

Figure 4.5. The effect of heavy metals on Nqo1 protein half-life. After cells were induced with 1 nM TCDD for 24 h, 10 μg/ml of CHX was added to inhibit further RNA synthesis, immediately before treatment with 5 μM As^{3+}, Cd^{2+}, or 25 μM Cr^{6+}. Protein was extracted at 0, 1, 3, 6, 12, and 24 h after incubation with the metals and subjected to Western blot analysis. The protein half-life values were determined from semilog plots of integrated densities versus time. * $p<0.05$, compared to the degradation of TCDD-induced Nqo1 protein.

concentrations. Interestingly, the induction in Nqo1 activity reached maximal induction at 1 nM, through there was no significant difference in Nqo1 activity between cells treated with 1 and 10 nM TCDD (Figure 4.6). Differential effects were noted when the cells were treated with TCDD in the presence of each metal. In contrast to its TCDD-concentration dependent potentiation of Nqo1 mRNA, As^{3+}, potentiated the induction of Nqo1 activity with low concentrations of TCDD, but significantly decreased its induction with 10 nM TCDD (Figure 4.6). The effect of Cd^{2+} and Cr^{6+} on Nqo1 activity, however, paralleled with their effects in Nqo1 mRNA levels. Cd^{2+} further potentiated the concentration-dependent induction of Nqo1 activity by TCDD while Cr^{6+} inhibited this induction with all concentrations of TCDD used (Figure 4.6). As expected based on the mRNA results, all three metals induced Nqo1 activity in vehicle-only treated cells (0 nM TCDD, Figure 4.6).

4.4.0.0. Discussion

Nqo1 is a detoxifying enzyme, catalyzing the two-electron reduction, and hence, detoxification of quinones (Joseph et al., 1994). An important regulator of intracellular redox status, Nqo1 protects against cellular oxidative stress and PAH-induced carcinogenicity (Long et al., 2000). Hence, tight regulation of this enzyme is of critical importance for protection against oxidative stress, macromolecule damage, and subsequent mutagenesis and carcinogenesis.

The results of this investigation verify the role of transcriptional mechanisms in the induction of Nqo1 by As^{3+} and Cd^{2+}. Although the exact mechanism(s) by which metals activate transcription have not been fully elucidated, a conglomerate of evidence generated from this and previous work in our lab may shed light on these mechanisms. Analysis of the murine Nqo1 gene reveals the presence of several cis acting elements that may have a role in the transcriptional regulation of Nqo1, including the ARE and the XRE. Induction of Nqo1 by TCDD requires functional AhR and Arnt, and an intact XRE (Ma et al., 2004, Prochaska and Talalay, 1988). However, we have previously shown that

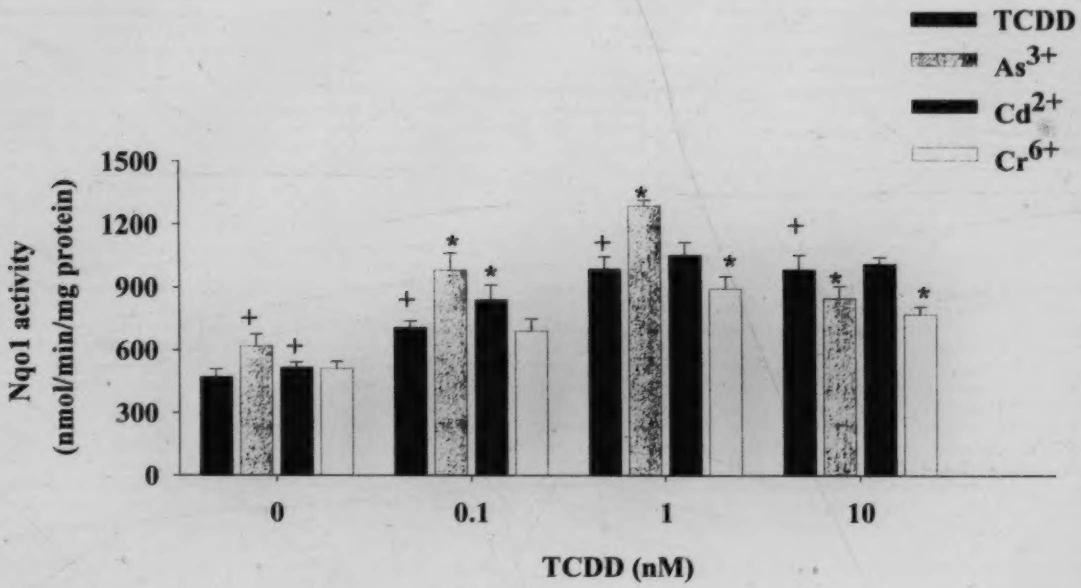

Figure 4.6. Effect of heavy metals on the concentration-dependent induction of Nqo1 activity. Cells were treated with 0 (vehicle), 0.1, 1, or 10 nM TCDD, in the presence and absence of 5 μM As^{3+}, Cd^{2+}, or 25 μM Cr^{6+} for 24 h. Nqo1 activity was measured in intact living cells using 7-ethoxyresorufin as a substrate. Values are presented as mean ± SEM (*n* = 8). + $p<0.05$, compared to control; * $p<0.05$, compared to respective TCDD treatment.

As^{3+} and Cd^{2+} are capable of inducing Nqo1 activity in AhR deficient Hepa 1c1c7 cells (chapter 2). Furthermore, as apparent from this study, As^{3+} is a more potent inducer of both Nqo1 mRNA than TCDD, verifying the importance of the ARE, and not the XRE, in the transcriptional induction of Nqo1 by the metals. Further to these findings, in this study, we provide evidence for a role of a labile protein in the constitutive Nqo1 expression and its induction by TCDD and As^{3+} and Cd^{2+}.

The role of the bZip transcription factor Nrf2 in directing gene activation through the ARE under conditions of oxidative stress is clearly established (Jaiswal, 2004, Ma et al., 2004, McMahon et al., 2001, Nioi and Hayes, 2004). Regulation of Nrf2 activity is controlled through subcellular localization and the total cellular level of the Nrf2 protein (Nioi and Hayes, 2004). Under non-stressful conditions, Nrf2 localizes in the cytoplasm by interacting with Kelch-like ECH associating protein 1 (Keap1) which in turn targets Nrf2 for degradation by the ubiquitin-26 S proteasome pathway (Itoh et al., 2003). Keap1 controls the ubiquitination of Nrf2 through the Cullin 3 (cul3)-dependent ubiquitin ligase (E3) (Kobayashi et al., 2004). Disruption of the Nrf2-Keap1 interaction, and hence activation of genes under the direction of the ARE, occurs during times of oxidative stress through multiple signaling pathways. Three potential pathways have been implicated in influencing Nrf2 signaling, the mitogen activated protein kinases (MAPKs) (Yu et al., 2000), protein kinase C (PKC) (Huang et al., 2000), and phosphatidylinositol 3-kinase (PI3K) (Kang et al., 2002). The majority of evidence, however, implicate the phosphorylation of Nrf2 by PKC in its liberation from Keap1 and its subsequent translocation into the nucleus

In the nucleus, Nrf2 dimerizes with c-Jun, Jun-B, Jun-D, or small Maf proteins, such as Maf K and Maf G, and binds to the ARE as a heterodimer, leading to the activation of Nqo1 and Gst a1 transcription (He et al., 2006, Jaiswal, 2004). With our results showing a decrease in metal-mediated induction of Nqo1 mRNA by CHX and the ability of MG-132 to protect against this decrease reaffirms the role of the Nrf2 system in the activation of Nqo1 transcription. These results are consistent with those obtained by other investigators. Arsenic activated Nrf2 in osteoblasts (Aono et al., 2003) and cultured keratinocytes (Pi et al., 2003), and He et al. confirmed the ability of As^{3+} to induce Nqo1 by disrupting the Nrf2-Keap1-Cul3 complex (He et al., 2006). Similarily, Cd^{2+} increased the half life of Nrf2 and subsequently induced heme oxygenase-1 (HO-1) expression (Stewart et al., 2003).

Unlike As^{3+} and Cd^{2+}, Cr^{6+} inhibited inducible Nqo1 expression. The mechanisms by which Cr^{6+} inhibits phase II enzyme expression may be multiple and various. Cr^{6+}-induced ROS is responsible for the activation of p53 (Wang et al., 2000) which has been shown to inhibit ARE-containing promoters (Faraonio et al., 2006). Interestingly, Cr^{6+} has also been previously shown to inhibit inducible, but not constitutive, gene expression by blocking the release of histone deacetylase (HDAC1) (Wei et al., 2004). The role of HDAC1 in the regulation of phase II enzymes is not known at this time. However, our results showing a more significant decrease in TCDD-mediated induction, but not constitutive Nqo1 mRNA, may be supportive of the hypothesis that Cr^{6+} inhibits inducible, but not constitutive, gene expression. However, the effect of Cr^{6+} on Gst a mRNA levels conflicts with this hypothesis. Cr^{6+} inhibited both inducible and constitutive Gst a mRNA levels, pointing to the role of multiple and various mechanisms in the modulation of Nqo1 and Gst a1 gene expression (chapter 2).

The posttranscriptional regulation of Nqo1 has not been extensively studied. Here, we provided the first evidence for posttranscriptional modifications of Nqo1 through changes in mRNA stabilization. All three metals seemingly decrease the mRNA half-life of the Nqo1 transcripts. However, the mechanism by which this happens still remains to be investigated. The decrease in Nqo1 mRNA stability during metal-induced oxidative stress could be a mechanism the body uses to control the expression of these enzymes during stress so as not to deplete its high energy resources.

In conclusion, the evidence provided here verifies the role of transcriptional mechanisms in the induction of phase II enzymes by As^{3+}, Cd^{2+}, and Cr^{6+}, likely through a labile transcription factor, in spite of posttranscriptional modifications. This study also demonstrates differential effects of heavy metals on the regulation of Nqo1 expression. Intense studies looking at the role of Nrf2 in mediating these effects are required.

4.5.0.0. References

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5.0.0.0. Chapter 5.0

THE ROLE OF OXIDATIVE STRESS IN THE MODULATION OF AHR-REGULATED GENES BY HEAVY METALS.

A version of this chapter has been published: Elbekai, R.H. and El-Kadi, A.O., 2005. *Free Radic Biol Med.* **39**, pp. 1499-511.

5.1.0.0. Introduction

We have previously shown that the heavy metals, arsenite (As^{3+}), cadmium (Cd^{2+}), and chromium (Cr^{6+}) inhibit AhR ligand-mediated induction of Cyp1a1 activity but potentiate the induction of Nqo1 and Gst a activities (chapter 2). Our studies have thus far implicated post-transcriptional mechanisms. However, the underlying precipitant(s) of metal effects is yet to be determined.

While each metal may have its own mechanisms of action, the induction of oxidative stress appears to be a common contributing mechanism by which metals may induce changes in the expression of AhR-regulated genes. As^{3+}, a well-recognized human carcinogen, has been shown to stimulate the production of O_2^- and H_2O_2 in various cellular systems, through the mitochondrial electron transfer chain, including human vascular smooth muscle cells (Lynn et al., 2000), human keratinocyte cells (Pi et al., 2003), human–hamster hybrid cells (Liu et al., 2001), and HEL30 cells (Corsini et al., 1999). Cd^{2+} exposure also induces oxidative stress in cultured human cells (Bagchi et al., 2000), likely by inhibiting the electron transfer chain (Wang et al., 2004), and increases malondialdehyde levels, an indicator of lipid peroxidation (Shaikh et al., 1999). Furthermore, Cd^{2+} has been shown to reduce the activity of antioxidant enzymes, including superoxide dismutase (Hussain et al., 1987) and catalase (Shaikh et al., 1999). Cr^{6+} has also been shown to induce oxidative stress, as evidenced by increased production of reactive oxygen species (ROS), nitric oxide production, and urinary excretion of malondialdehyde (Bagchi et al., 1995) and (Hassoun and Stohs, 1995). Through a Haber-Weiss-type reaction, Cr^{6+} is reduced by O_2^- when it enters the biological system and then reacts with H_2O_2 to generate $^{\bullet}OH$ and OH^- (Leonard et al., 2004).

ROS have been shown to play a role in the decrease in CYP450 in vivo (Galal and Souich, 1999) and in vitro (Elbekai et al., 2004), an effect that is modulated by changes in cellular redox potential (Barker et al., 1994). Phase II xenobiotic-metabolizing enzymes are also sensitive to oxidative stress. Nqo1 is a redox-sensitive gene that is part of a cellular defense mechanism responsible for the induction of more than two dozen genes in response to oxidative stress (Radjendirane et al., 1997). In vitro studies have demonstrated an increase in GST mRNA and protein as a result of cell membrane lipid peroxidation (Tjalkens et al., 1998a). In vivo studies have also shown iron-induced lipid peroxidation to cause an induction in GST, in particular the α-class (Tjalkens et al., 1998b).

Based on the pool of evidence from previous studies, we hypothesize that As^{3+}-, Cd^{2+}-, or Cr^{6+}-induced oxidative stress plays a role in disrupting the expression of AhR-regulated genes. We also hypothesize that up-regulation or down-regulation of intracellular glutathione (GSH) can inhibit or potentiate metal-induced modulation of AhR-regulated genes. To test these hypotheses, we investigated the ability of the heavy metals to induce oxidative stress. We also tested the effect of a prooxidant, buthionine-(*S,R*)-sulfoximine (BSO) and an antioxidant, *N*-acetylcysteine (NAC), on the modulation of AhR-regulated gene expression by the heavy metals.

5.2.0.0. Materials and methods

Sodium arsenite, l-buthionine-(*S,R*)-sulfoximine, cadmium chloride, chromium trioxide, 2′,7′-dichlorofluorescein diacetate, 2,6-dichlorophenolindophenol, 5,5′-dithiobis-(2-nitrobenzoic acid), 7-ethoxyresorufin, fluorescamine, and *N*-acetylcysteine were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent and the random primers DNA labeling system were purchased from Invitrogen Co. (Grand Island, NY). Hybond-N nylon membranes were from Amersham Canada (Oakville, ON). [α-^{32}P]dCTP (3000 Ci/mmol) was supplied by the DNA Core Services Laboratory, University of Alberta. All other chemicals were purchased from Fisher Scientific (Toronto, ON).

5.2.1.0. Cell culture

Hepa 1c1c7 cell lines, generously provided by Dr. O. Hankinson at the University of California (Los Angeles, CA), were maintained in Dulbecco's modified Eagle's medium (DMEM), without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 µM l-glutamine, 50 µg/ml gentamycin sulfate, 100 IU/ml penicillin, 10 µg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM nonessential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37°C in a 5% CO_2 humidified incubator.

5.2.2.0. Chemical treatments

Cells were treated in serum-free medium alone or with 1 nM of the AhR ligand TCDD dissolved in DMSO, metals dissolved in double deionized water, or both. The various metals were added 30 min prior to the addition of TCDD, when applicable. TCDD was maintained in DMSO at −20°C until use but the metals were prepared freshly for each treatment. In all treatments, the concentration of double de-ionized water in treatment medium did not exceed 0.5% (v/v) while the DMSO concentration did not exceed 0.05% (v/v). For enzyme activity assays, the duration of chemical exposure was 24 h. However, RNA was extracted only 6 h after chemical treatment. When applicable, 100 µM BSO or 10 mM NAC, which were used to modify the condition of oxidative stress, was added 24 or 6 h prior to the addition of the metals, respectively.

5.2.3.0. Preparation of cellular homogenates

Cells were grown for 48 h in 6-well plates before treatment with TCDD and/or metals. After the elapsed treatment period, cells were washed with PBS and kept in 0.5 ml of homogenization buffer, in a −80°C freezer, for at least 24 h. Thawed cells were extracted and homogenized with a Kontes homogenizer and then centrifuged at 10,000g for 20 min. The supernatant fractions were then removed and protein concentrations in the supernatant were determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as the standard. The extracted cellular homogenates were stored in a −20°C freezer until they were used for determination of Nqo1 enzyme activity and GSH content.

5.2.4.0. Determination of ROS

To determine the degree of ROS generation induced by the metals, a fluorometric assay, utilizing the unique intracellular oxidation of 2′,7′-dichlorofluorescein diacetate (DCF-DA), was used. Cells grown for 24 h in 96-well plates were pretreated with 5 μM DCF-DA 2 h prior to the addition of the metals. After 24 h incubation with the metals in the dark, fluorescence was measured using the Baxter 96-well plate reader using excitation and emission wavelengths of 485 and 535 nm, respectively.

5.2.5.0. Glutathione analysis

Glutathione content was determined by the 5,5′-dithiobis-(2-nitrobenzoic acid)-glutathione disulfide (DTNB-GSSG) reductase recycling assay (Anderson and Meister, 1983). The amount of 0.01 mg of cell homogenate protein was added to a solution of sodium phosphate buffer containing 0.3 mM NADPH and 0.6 mM DTNB, warmed to 30°C. The assay was initiated with the addition of 2 units of GSSG reductase. The formation of 5-thio-2-nitrobenzoic acid (TNB) from DTNB was followed spectrophotometrically at 412 nm. Glutathione content was determined using standard curves generated with known amounts of glutathione.

5.2.6.0. Determination of EROD activity

The Cyp1a1-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity assay was performed on intact, living cells as described previously (chapter 2). Enzymatic activity was normalized for cellular protein content which was determined using a modified fluorescent assay (chapter 2).

5.2.7.0. Determination of Nqo1 activity

Nqo1 activity was determined by the continuous spectrophotometric assay of Ernster (Ernster, 1967), which quantitates the reduction of 2,6-dichlorophenolindophenol (DCPIP) by 0.01 mg of cell homogenate protein in the presence of β-nicotinamide adenine dinucleotide phosphate (NADPH; 200 μM) and flavin adenine dinucleotide

(FAD; 5 µM). The rate of reduction of DCPIP (40 µM) in 1 ml of Tris-HCl buffer (pH 7.8, 25 mM) containing 0.1% (v/v) Tween 20 and 0.023% bovine serum albumin was monitored for 90 s at 600 nm using an extinction coefficient of 2.1 mM^{-1} cm^{-1}.

5.2.8.0. RNA extraction and analysis.

Total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen Co). Northern blot analysis of total RNA was performed as described elsewhere (chapter 1). The cDNA probes for mouse Cyp1a1, heme oxygenase-1 (HO-1), and Gapdh mRNA were generously provided by Dr. John R. Bend (University of Western Ontario, London, ON). The cDNA probe for mouse Gst a mRNA was provided by Dr. David Eaton (University of Washington, Seattle, WA). The mouse Nqo1 cDNA probe was prepared as previously described (Chen et al., 1994). All probes were ^{32}P-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions.

5.2.9.0. Statistical analysis

All results are presented as mean ± SE. The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc comparison. The differences were considered significant when $p < 0.05$.

5.3.0.0. Results

5.3.1.0. Oxidative stress induced by metal exposure

We investigated whether heavy metals could trigger oxidative stress in Hepa 1c1c7 cells by measuring intracellular ROS based on quantification of the oxidation-activated fluorescent dye DCF-DA, determining the cellular GSH status, and quantifying HO-1 mRNA levels. The oxidation of DCF to its fluorescent probe was measured 24 h after cells were treated with As^{3+} (1–10 µM), Cd^{2+} (1–25 µM), or Cr^{6+} (1–25 µM) (Figure 5.1A). Only Cd^{2+} and Cr^{6+}, at the highest concentration tested (25 µM), caused a significant increase in DCF fluorescence. As^{3+}, at all concentrations tested, failed to produce an increase in the production of ROS, as shown by the lack of DCF fluorescence (Figure 5.1A). Also, exposure to As^{3+} (1–10 μM), Cd^{2+} (1–5 μM), or Cr^{6+} (1–25 μM) produced significant increases in cellular GSH, although the increase did not appear to be concentration dependent (Figure 5.1B). However, Cd^{2+}, at the highest concentration tested (25 μM), caused a significant reduction in GSH levels. In addition, we measured the level of HO-1 mRNA, a sensitive marker of oxidative stress (Poss and Tonegawa, 1997). Not surprisingly, exposing cells to 5 μM As^{3+}, Cd^{2+}, or Cr^{6+} alone (Figure 5.2A) or cotreated with TCDD (Figure 5.3A) significantly induced HO-1 mRNA levels. As^{3+} was the most potent inducer of HO-1 mRNA (about 1500% increase) with Cd^{2+} being the second most potent and Cr^{6+} the least potent.

For the remainder of our experiments, the induction of HO-1 mRNA was used as a positive control of the cellular stress response induced by the metals. The concentration of metals used in these studies (5 μM) was chosen after determining the ability of a range of concentrations to modulate AhR-regulated gene expression (chapter 2) without significantly affecting Hepa 1c1c7 cell viability in the presence of BSO.

5.3.2.0. Superinduction of HO-1 mRNA in cells pretreated with BSO and stimulated with heavy metals

To deplete intracellular GSH, we treated cells with BSO, a potent and specific inhibitor of γ-glutamylcysteine synthetase. Exposing cells to BSO alone did not appear to cause any significant induction in HO-1 mRNA. However, the induction of HO-1 mRNA by the metals alone (Figure 5.2A) or in the presence of TCDD (Figure 5.3A) was further potentiated in cells pretreated with BSO. This observation confirms the role of oxidative stress in the regulation of HO-1 expression and supports the use of BSO for the amplification of the state of oxidative stress induced by the metals.

5.3.3.0. Superinduction of AhR-regulated genes' mRNA levels in cells pretreated with BSO and stimulated with heavy metals

All three metals induced AhR-regulated genes at the mRNA level alone (Figure 5.2B) and potentiated their induction by TCDD (Figure 5.3B). At the constitutive level, As^{3+}

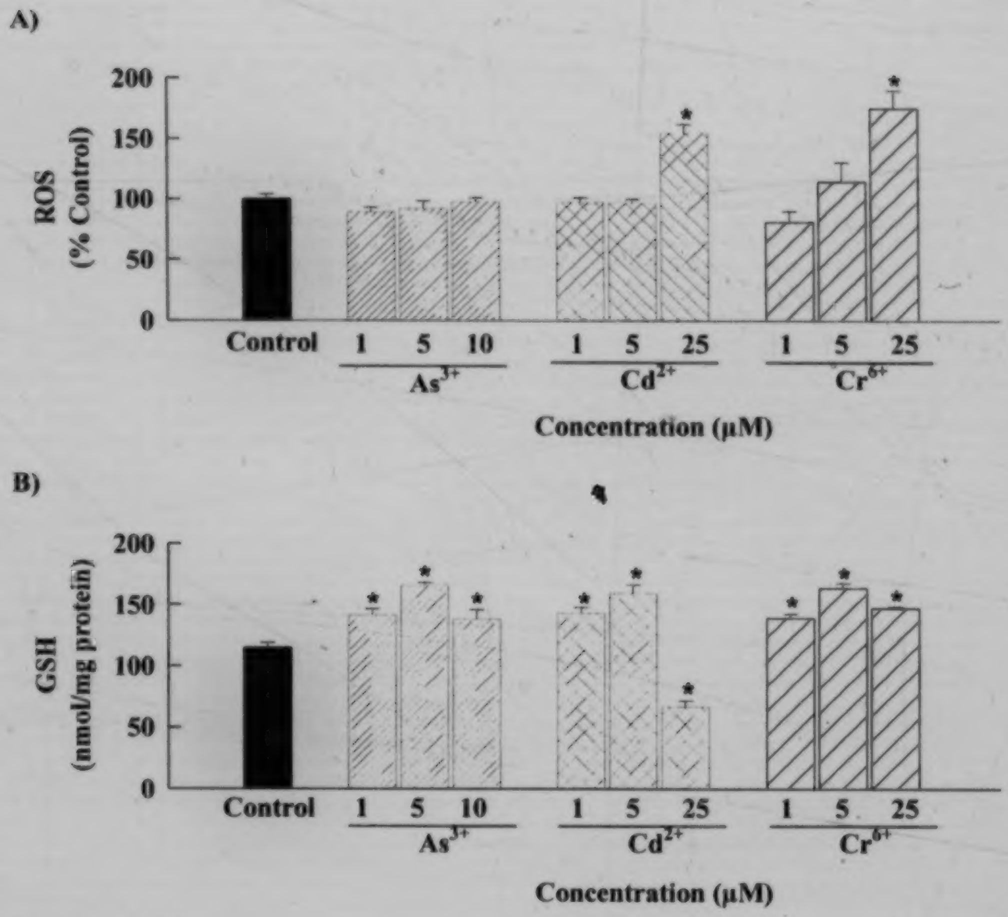

Figure 5.1. Induction of oxidative stress as measured by the formation of the fluorescent form of DCF (A) and the concentration of cellular GSH (B). For the DCF-DA assay, Hepa 1c1c7 cells were exposed to increasing concentrations of As^{3+} (1–10 μM), Cd^{2+} (1–25 μM), or Cr^{6+} (1–25 μM) for 24 h in the presence of DCF-DA. Fluorescence was then measured using excitation and emission wavelengths of 485 and 535 nm, respectively. Data are expressed as mean fluorescence intensity/mg protein ± SE (% Control) where $n = 8$. To determine cellular GSH levels, cells were exposed to increasing concentrations of As^{3+} (1–10 μM), Cd^{2+} (1–25 μM), or Cr^{6+} (1–25 μM) for 24 h before extraction of the cytosolic fraction. Cellular GSH was determined by measuring the conversion of DTNB to TNB, spectrophotometrically. Data are expressed as mean concentration/mg protein ± SE where $n = 6$. *$p < 0.05$ compared to control (non-metal-exposed).

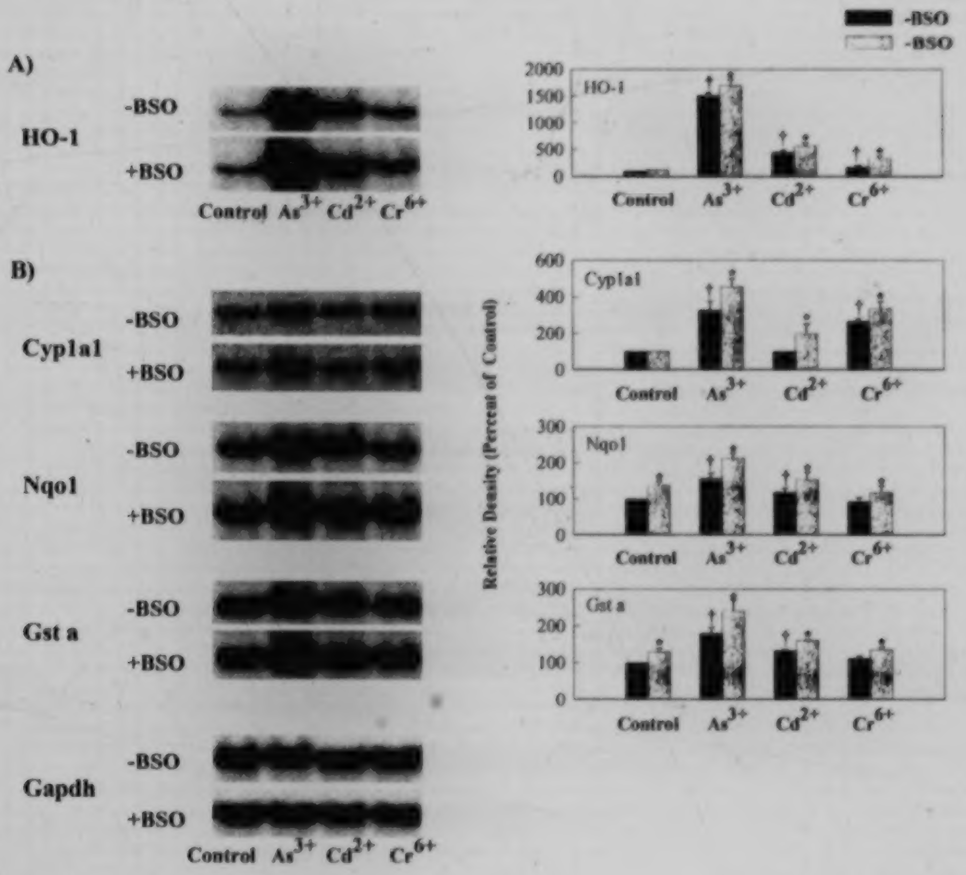

Figure 5.2. The effect of BSO pretreatment on heavy metal-mediated inductions of HO-1 (A) and Cyp1a1, Nqo1, and Gst a (B) mRNA in Hepa 1c1c7 cells. Cells were exposed to 5 µM As^{3+}, Cd^{2+}, or Cr^{6+} for 6 h. When applicable, cells were pretreated with 100 µM BSO for 24 h, prior to the addition of the metals. Total RNA was extracted using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a ^{32}P-labeled cDNA probe. Northern blots were performed at least three times with similar observations using different samples but only one representative result is shown. Gapdh was used as a loading control. The graphs demonstrate mRNA levels, as quantified by densitometry. Error bars represent SE of three independent experiments. $^{\dagger}p < 0.05$, compared to control; $^{*}p < 0.05$, +BSO treatments compared to respective –BSO treatments.

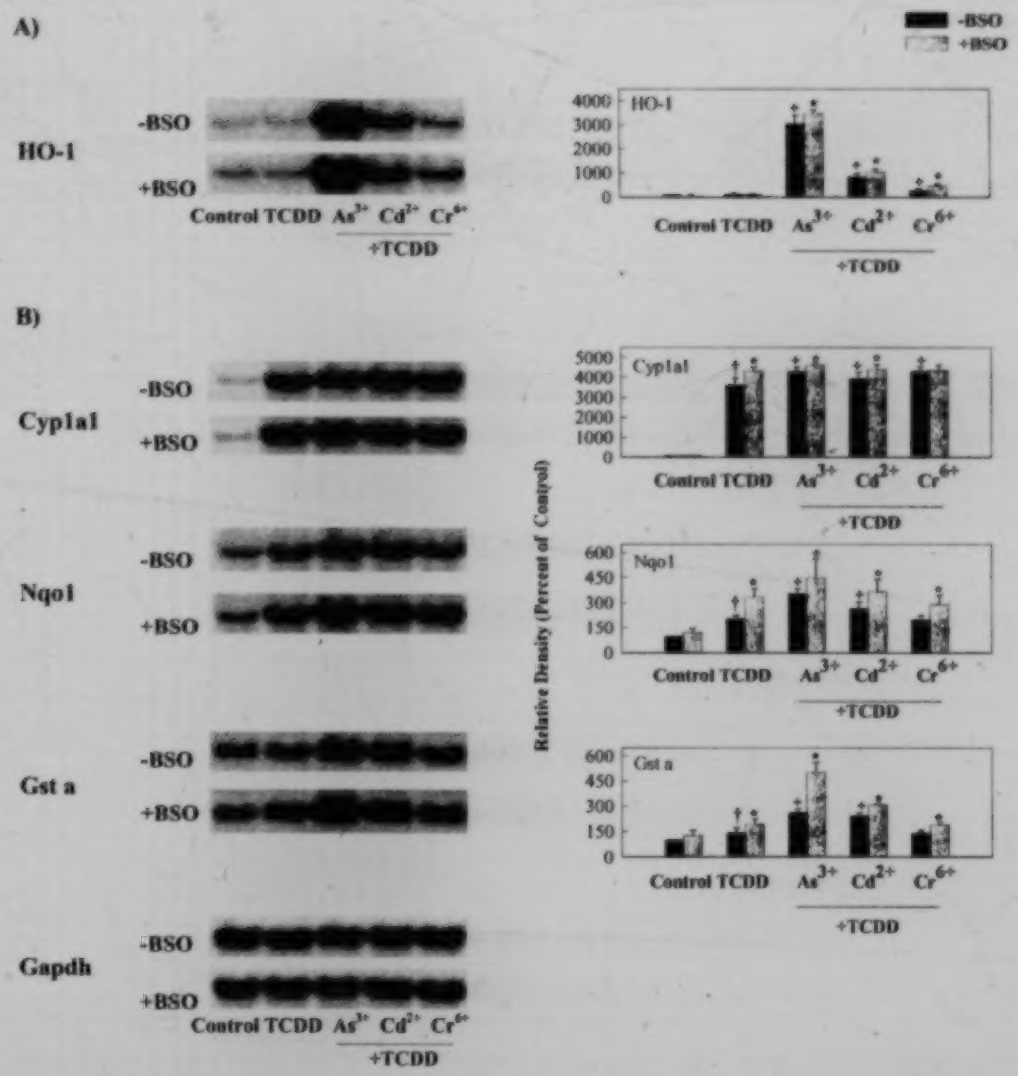

Figure 5.3. The effect of BSO pretreatment on heavy metal-mediated inductions of HO-1 (A) and Cyp1a1, Nqo1, and Gst a (B) mRNA in Hepa 1c1c7 cells in the presence of TCDD. Cells were exposed to 1 nM TCDD with 5 μM As^{3+}, Cd^{2+}, or Cr^{6+} for 6 h. When applicable, cells were pretreated with 100 μM BSO for 24 h, prior to the addition of the metals. Total RNA was extracted using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a ^{32}P-labeled cDNA probe. Northern blots were performed at least three times with similar observations using different samples but only one representative result is shown. Gapdh was used as a loading control. The graphs demonstrate mRNA levels, as quantified by densitometry. Error bars represent SE of three independent experiments. $^{\dagger}p < 0.05$, compared to control; $^{+}p < 0.05$, compared to TCDD only; $^{*}p < 0.05$, +BSO treatments compared to respective –BSO treatments.

caused the greatest induction in Cyp1a1 mRNA, while both As^{3+} and Cr^{6+} were equally potent at potentiating TCDD-induced Cyp1a1 mRNA induction. While BSO alone did not alter Cyp1a1 mRNA expression, it significantly potentiated the induction by all three metals, alone (Figure 5.2B) and in the presence of TCDD (Figure 5.3B). The presence of BSO also enhanced Cyp1a1 mRNA induction by TCDD alone (Figure 5.3B).

We also examined the effect of glutathione depletion on the modulation of Nqo1 and Gst a mRNA levels by As^{3+}, Cd^{2+}, and Cr^{6+}. Not surprisingly, As^{3+} or Cd^{2+} alone significantly induced Nqo1 and Gst a mRNA levels (Figure 5.2B) and potentiated their induction by TCDD (Figure 5.3B). As anticipated, pretreatment with BSO superinduced the induction of Nqo1 and Gst a mRNA by the two metals in the absence and presence of TCDD. These observations corroborate a role for oxidative stress in the induction of Nqo1 and Gst a by As^{3+} and Cd^{2+}. Cr^{6+}, on the other hand, had no significant effect on Nqo1 or Gst a mRNA levels in cells treated with the metal alone, or in the presence of TCDD (Figure 5.2 and Figure 5.3). However, in the presence of BSO, Cr^{6+} alone increased Nqo1 and Gst a mRNA levels by 126 and 121%, respectively. In the presence of TCDD and BSO, Cr^{6+} caused a 147 and 132% induction in Nqo1 and Gst a mRNA levels, respectively. Not surprisingly, BSO alone significantly induced the expression of Nqo1 and Gst a at the mRNA level (Figure 5.2 and Figure 5.3).

5.3.4.0. BSO potentiates the effect of metals on AhR-regulated genes at the activity level

Further to our attempt to define the level of regulation of AhR-regulated genes by oxidative stressors, we tested the effect of BSO on the modulation of AhR-regulated genes by heavy metals at the activity level. Cotreatment of Hepa 1c1c7 cells with metals and TCDD elicited significant inhibition of TCDD-dependent induction of EROD activity at the 5 μM concentration, an effect which contradicted Cyp1a1 mRNA results (Figure 5.4). In addition, these effects were potentiated significantly in the presence of BSO. This was most notable with As^{3+}, as pretreatment with BSO reduced EROD activity

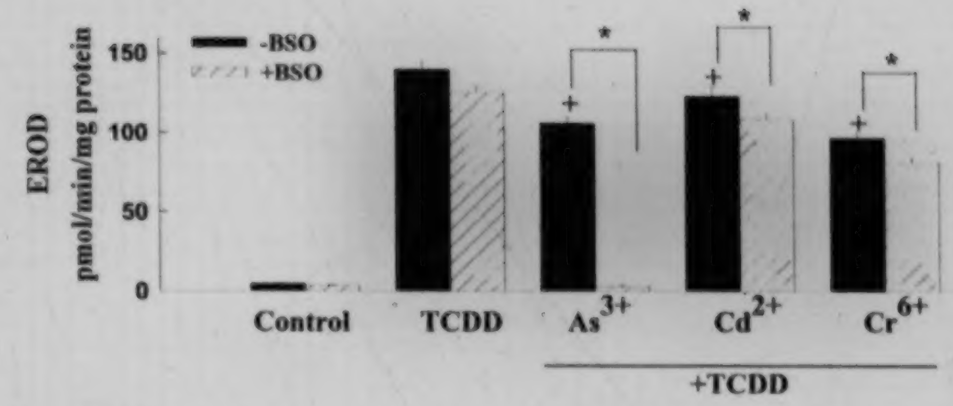

Figure 5.4. Effect of BSO on As^{3+}-, Cd^{2+}-, and Cr^{6+}-mediated inhibition of EROD activity in Hepa 1c1c7 cells. Cells were treated for 24 h with 5 μM As^{3+}, Cd^{2+}, or Cr^{6} in the presence of 1 nM TCDD. When applicable, cells were pretreated with 100 μM BSO for 24 h, prior to the addition of the metals. EROD activity was measured fluorometrically, as described under Materials and methods. Values represent mean activity ± SE where $n = 8$. $^{+}p < 0.05$, compared to TCDD only; $^{*}p < 0.05$, +BSO treatments compared to respective –BSO treatments.

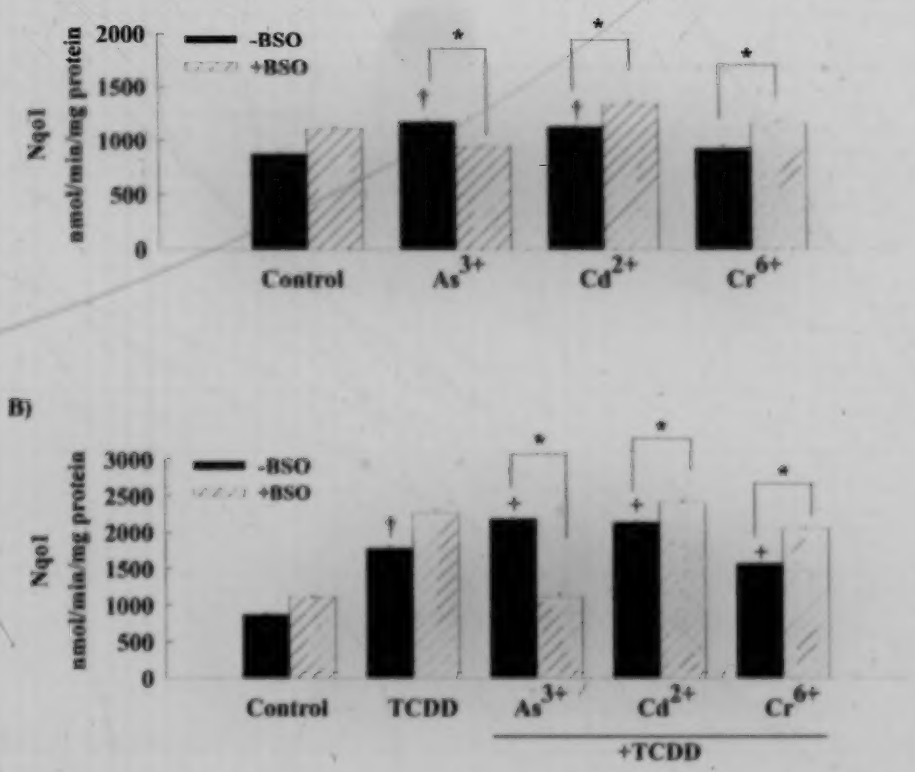

Figure 5.5. Effect of BSO on As^{3+}-, Cd^{2+}-, and Cr^{6+}-mediated induction of constitutive (A) and inducible (B) Nqo1 activity in Hepa 1c1c7 cells. Cells were treated for 24 h with 5 μM As^{3+}, Cd^{2+}, or Cr^{6+} in the presence and absence of 1 nM TCDD. When applicable, cells were pretreated with 100 μM BSO for 24 h, prior to the addition of the metals. Cellular homogenates were used to determine Nqo1 activity spectrophotometrically, as described under Materials and methods. Data are expressed as mean ± SE where $n = 6$. †$p < 0.05$ compared to control, $^{+}p < 0.05$ compared to TCDD only, $^{*}p < 0.05$ +BSO treatments compared to respective –BSO treatments.

to a level that was not significantly different from the activity measured in control, untreated cells (Figure 5.4). On the other hand, an excellent correlation was observed between Nqo1 mRNA and activity levels. Both As^{3+} and Cd^{2+} significantly induced Nqo1 activity alone (Figure 5.5A) and potentiated the induction mediated by TCDD (Figure 5.5B). Furthermore, pretreatment with BSO further enhanced the effect of Cd^{2+} on Nqo1 activity (Figure 5.5). However, in contrast to what is expected based on mRNA results, glutathione depletion repressed the inductive capacity of As^{3+} on Nqo1 activity, at the constitutive and inducible levels (Figure 5.5). In the presence of BSO, Nqo1 activity in cells treated with As^{3+}, alone or with TCDD, was not significantly different than activity in cells treated with BSO only. Also, Cr^{6+} alone had no significant effect on Nqo1 activity. However, in the presence of BSO, Cr^{6+} significantly induced Nqo1 activity (Figure 5.5A). On the other hand, Cr^{6+} inhibited TCDD-mediated induction of Nqo1 activity, although BSO partially protected against this decrease (Figure 5.5B).

5.3.5.0. Inhibition of HO-1 mRNA levels by the antioxidant NAC in cells stimulated with heavy metals

In the present study, we also tested whether replenishing thiols would decrease metal-mediated induction of HO-1 mRNA levels in Hepa 1c1c7 cells. For this purpose, cells were pretreated with 10 mM of the thiol antioxidant NAC before the addition of the metals and/or TCDD. While having no significant effect on its own, NAC dramatically reduced the levels of HO-1 mRNA in cells treated with heavy metals with and without TCDD (Figure 5.6 and Figure 5.7). In metal-only treated cells, NAC reduced HO-1 mRNA induction by 2-, 6-, and 4-fold with As^{3+}, Cd^{2+}, and Cr^{6+}, respectively (Figure 5.6A). In addition, NAC decreased HO-1 mRNA levels by 3-, 17-, and 6-fold in cells treated with TCDD and As^{3+}, Cd^{2+}, or Cr^{6+}, respectively (Figure 5.7A). These findings clearly show that oxidative stress induced by heavy metals can be lessened by NAC.

5.3.6.0. Inhibition of metal-mediated induction of AhR-regulated genes by NAC at the mRNA level in cells stimulated with heavy metals

As discussed previously, all three metals up-regulated the expression of AhR-regulated genes at the mRNA level, alone (Figure 5.2B), and in the presence of TCDD (Figure 5.3B). However, while having no significant effect on its own, NAC diminished metal-mediated induction of the mRNA levels of all genes tested, at the constitutive level (Figure 5.6B). The most significant decrease was in cells treated with As^{3+} as there was a 3.5-, 1.5-, and 1.7-fold decrease in Cyp1a1, Nqo1, and Gst a mRNA levels, respectively, in cells treated with As^{3+}+NAC compared to cells treated with As^{3+} alone (Figure 5.6B). On the other hand, Cd^{2+} alone and in the presence of NAC did not alter Cyp1a1 mRNA expression.

The effect of NAC was less obvious in cells treated with metals in the presence of TCDD (Figure 5.7B), which may indicate protection by NAC of metal- but not TCDD-mediated induction of AhR-regulated genes. The potentiation of Cyp1a1 mRNA levels by the three metals was not significantly altered with NAC (Figure 5.7B). However, replenishing thiols minimized the potentiation of Nqo1 and Gst a mRNA by As^{3+} and Cd^{2+}.

Cr^{6+} did not alter Nqo1 and Gst a mRNA levels in the absence of TCDD but inhibited Gst a mRNA levels in the presence of TCDD. However, in the presence of TCDD+NAC, Cr^{6+} induced Gst a mRNA levels, when compared to cells treated with TCDD+NAC only (Figure 5.7B).

5.3.7.0. NAC protects against metal-induced modulation of AhR-regulated genes at the activity level

NAC completely prevented As^{3+}- and Cd^{2+}-mediated inhibition of TCDD-dependent induction of EROD activity. With Cr^{6+}, NAC not only prevented the decrease in EROD activity, but it also increased EROD activity by 1.4-fold compared to cells treated with Cr^{6+}+TCDD (Figure 5.8). In addition, NAC diminished Nqo1 activity induction by As^{3+} or Cd^{2+} alone without having a significant effect on its own (Figure 5.9A). Cr^{6+} alone, in the presence and absence of NAC, had no significant effect on Nqo1 activity levels.

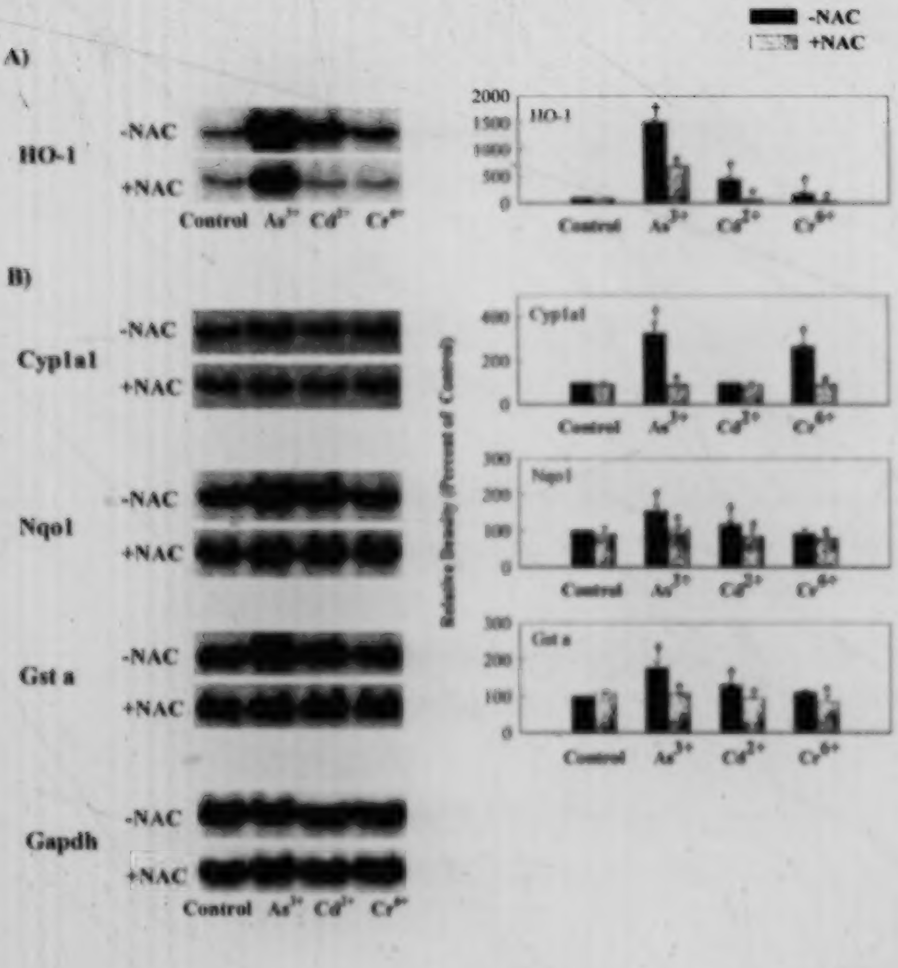

Figure 5.6. The effect of NAC pretreatment on heavy metal-mediated inductions of HO-1 (A) and Cyp1a1, Nqo1, and Gst a (B) mRNA in Hepa 1c1c7 cells. Cells were exposed to 5 μM As^{3+}, Cd^{2+}, or Cr^{6+} for 6 h. When applicable, cells were pretreated with NAC for 6 h, prior to the addition of the metals. Total RNA was extracted using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a ^{32}P-labeled cDNA probe. Northern blots were performed at least three times with similar observations using different samples but only one representative result is shown. Gapdh was used as a loading control. The graphs demonstrate mRNA levels, as quantified by densitometry. Error bars represent SE of three independent experiments. $^{\dagger}p < 0.05$, compared to control; $^{*}p < 0.05$, +NAC treatments compared to respective –NAC treatments.

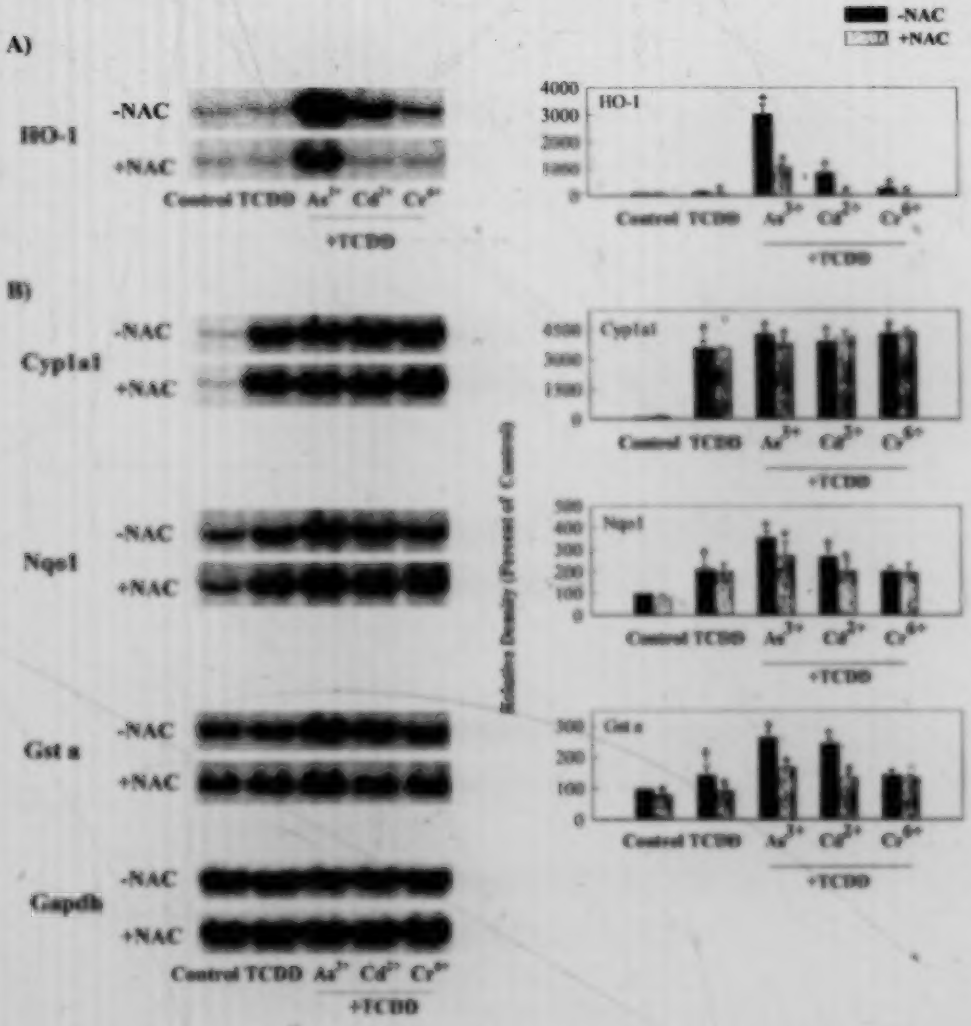

Figure 5.7. The effect of NAC pretreatment on heavy metal-mediated inductions of HO-1 (A) and Cyp1a1, Nqo1, and Gst a (B) mRNA in Hepa 1c1c7 cells in the presence of TCDD. Cells were exposed to 1 nM TCDD with 5 μM As^{3+}, Cd^{2+}, or Cr^{6+} for 6 h. When applicable, cells were pretreated with NAC for 6 h, prior to the addition of the metals. Total RNA was extracted using TRIzol reagent, separated on a 1.1% formaldehyde denaturing gel, transferred to Hybond-N nylon membranes, and hybridized with a ^{32}P-labeled cDNA probe. Northern blots were performed at least three times with similar observations using different samples but only one representative result is shown. Gapdh was used as a loading control. The graphs demonstrate mRNA levels, as quantified by densitometry. Error bars represent SE of three independent experiments. $^{\dagger}p < 0.05$, compared to control; $^{+}p < 0.05$, compared to TCDD only; $^{*}p < 0.05$, +NAC treatments compared to respective –NAC treatments.

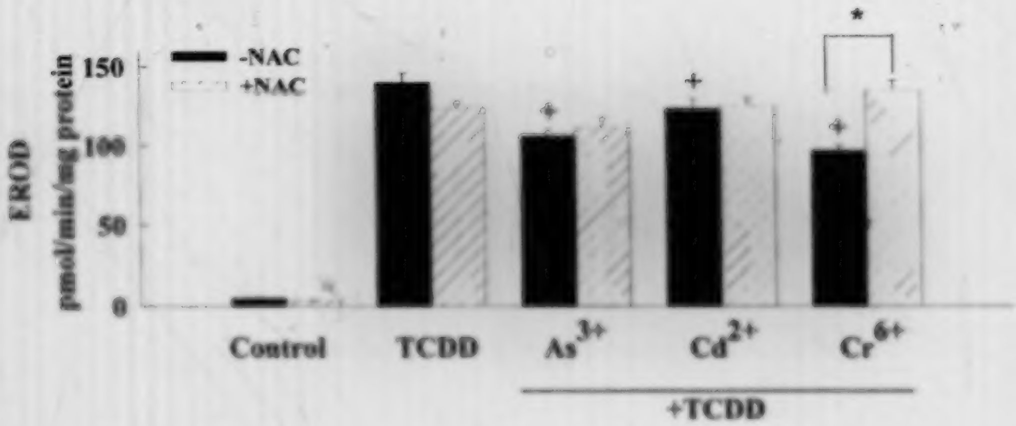

Figure 5.8. Effect of NAC on As^{3+}-, Cd^{2+}-, and Cr^{6+}-mediated inhibition of EROD activity in Hepa 1c1c7 cells. Cells were treated for 24 h with 5 μM As^{3+}, Cd^{2+}, or Cr^{6} in the presence of 1 nM TCDD. When applicable, cells were pretreated with 10 mM NAC for 6 h, prior to the addition of the metals. EROD activity was measured fluorometrically, as described under Materials and methods. Values represent mean activity ± SE where $n = 8$. $^{+}p < 0.05$, compared to TCDD only; $^{*}p < 0.05$, +NAC treatments compared to respective –NAC treatments.

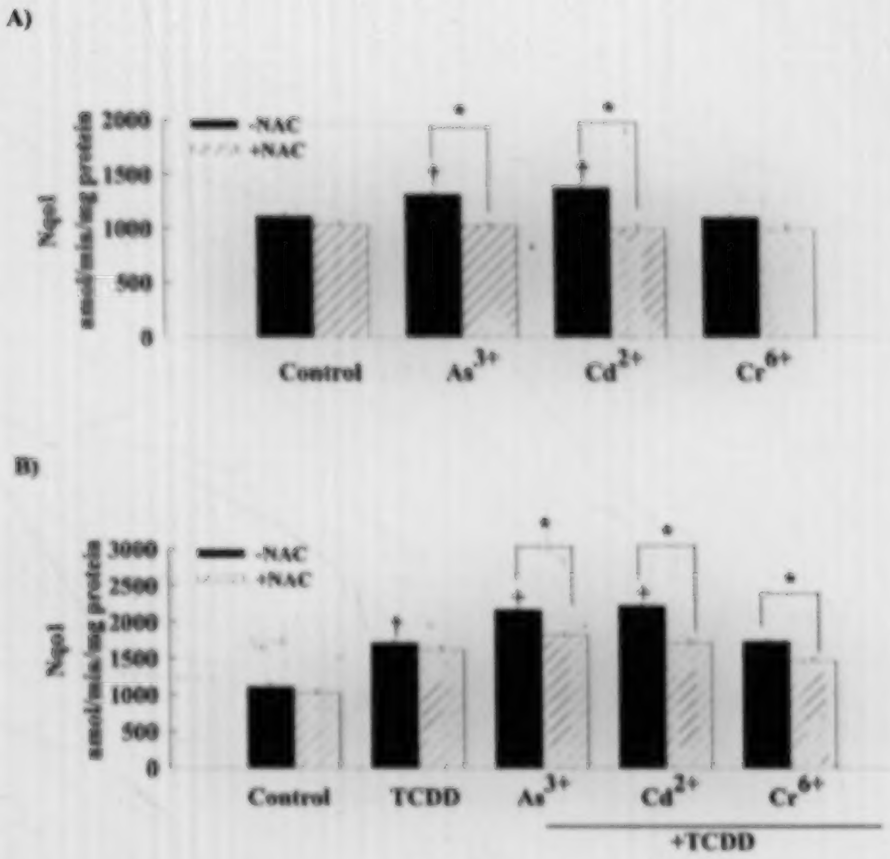

Figure 5.9. Effect of NAC on As^{3+}-, Cd^{2+}-, and Cr^{6+}-mediated mediated induction of constitutive (A) and inducible (B) Nqo1 activity in Hepa 1c1c7 cells. Cells were treated for 24 h with 5 μM As^{3+}, Cd^{2+}, or Cr^{6+} in the presence and absence of 1 nM TCDD. When applicable, cells were pretreated with 10 mM NAC for 6 h, prior to the addition of the metals. Cellular homogenates were used to determine Nqo1 activity spectrophotometrically, as described under Materials and methods. Data are expressed as mean ± SE where $n = 6$. $^{\dagger}p < 0.05$, compared to control; $^{+}p < 0.05$, compared to TCDD only; $^{*}p < 0.05$, +NAC treatments compared to respective –NAC treatments.

Similarly, NAC diminished any contribution of As^{3+} and Cd^{2+} on Nqo1 activity induction in cells treated with metals and TCDD, without altering TCDD-mediated induction of Nqo1 activity. Also, NAC reduced Nqo1 activity in cells treated with Cr^{6+}+TCDD to below TCDD-induced levels (Figure 5.9B).

5.4.0.0. Discussion

Our previous report (chapter 2) showing that the heavy metals As^{3+}, Cd^{2+}, and Cr^{6+} affect the induction of Cyp1a1, Nqo1, and Gst a prompted us to investigate the mechanism of this interaction, to enhance our understanding of how these metals could alter the mutagenicity of AhR ligands. In particular, we designed studies to examine the role of metal-induced oxidative stress on the expression of these AhR-regulated genes. We initially confirmed the ability of the metals to induce oxidative stress in Hepa 1c1c7 cells using three oxidative stress markers: the production of intracellular ROS, cellular glutathione content, and the induction of HO-1 mRNA levels. Next, we determined the effect of manipulating cellular redox status on the modulation of AhR-regulated genes, at the mRNA and activity levels, by the heavy metals.

In the present study, we found that only Cd^{2+} and Cr^{6+} increased the production of ROS, which is in agreement with previously published results (Sugden et al., 2004). To further examine whether metals induce oxidative stress, we determined cellular GSH concentration. GSH, the most abundant redox balance buffer in vivo, protects the cell against endogenous and exogenous oxidative stress by reacting with ROS to generate GSSG (Wu et al., 2004). As^{3+} (1–10 μM), Cd^{2+} (1–5 μM), and Cr^{6+} (1–25 μM) increased GSH levels, which is in agreement with previously reported studies (Schuliga et al., 2002, Shukla et al., 2000, Yeh et al., 2002). The increase in GSH may be part of the cellular stress response against the presence of ROS. When cells are exposed to high concentrations of ROS, GSH synthesis increases initially to facilitate the removal of lipid peroxidation products (Schuliga et al., 2002). This is an adaptive cellular response mediated by the induction of Gst isoenzymes, as demonstrated in our results. Of interest, Cd^{2+} significantly reduced GSH at the highest concentration (25 μM). A decrease in GSH levels after Cd^{2+} exposure has been reported previously (Ercal et al., 2001) and is likely due to a dramatic increase in GSSG levels and subsequent inhibition of GSSG reductase, resulting in a decrease in GSH regeneration.

To further confirm that heavy metals induce oxidative stress in Hepa 1c1c7 cells, we examined the effect of the metals on HO-1 mRNA levels. In the present study we found that all metals increased HO-1 mRNA levels. HO, an enzyme of 32 kDa, catalyzes the oxidative conversion of heme into biliverdin which serves an important role in protecting cells from oxidative damage, such as free radicals (Marilena, 1997). The HO-1 isozyme, the major isoform, is inducible in response to oxidative stimuli and offers protection against further cellular injury in the presence of ROS (Takahashi et al., 2004).

To verify the role of oxidative stress in the modulation of AhR-regulated genes, glutathione redox status was altered and the AhR-regulated genes' mRNA and activity levels were measured. When cellular GSH is depleted, cells become extremely vulnerable to oxidative damage. Hence, with the use of a prooxidant, we expected to see an enhancement of the metal effects on the expression of Cyp1a1, Nqo1, and Gst a. In parallel, we also used an antioxidant to minimize the cellular stress response. NAC's antioxidant properties are believed to originate from its ability to stimulate glutathione synthesis, therefore maintaining intracellular GSH levels and scavenging ROS (Flora, 1999).

The reduction of TCDD-induced Cyp1a1-specific EROD activity by heavy metals has been clearly established (Vakharia et al., 2001). However, the mechanisms through which such inhibition occurs remain very controversial. Many reports have concluded that transcriptional mechanisms are involved in the inhibition of CYP1A1 expression in human HepG2 cells (Bessette et al., 2005). However, these mechanisms appear to be cell specific. As^{3+} decreased benzo[*a*]pyrene-mediated induction of CYP1A1 activity in T-47D cells without altering the mRNA levels (Spink et al., 2002). Also, we have reported potentiations in Cyp1a1 mRNA by As^{3+}, Cd^{2+}, and Cr^{6+} which were not consistent with an inhibition in TCDD-mediated induction of EROD activity. Moreover, the prooxidant BSO potentiated the increase in Cyp1a1 mRNA levels by the metals in the absence and presence of TCDD while the antioxidant NAC protected against such increase. On the other hand, BSO significantly potentiated the decrease in Cyp1a1-specific EROD activity by all three metals while NAC prevented the inhibition of TCDD-induced EROD activity. Hence, there appears to be two distinct mechanisms through which metals increase Cyp1a1 mRNA but decrease its activity expression. Nevertheless, ROS seem to be involved in the modulation of this gene at both the mRNA and activity levels.

In light of our new data, it is more plausible to conclude that ROS play a role in the induction of Cyp1a1 mRNA levels through transcriptional mechanisms. Many previously published reports emphasize the role of ROS in activating transcription factors and inducing gene transcription. The mechanisms by which ROS may up-regulate Cyp1a1 mRNA remain unclear, but many postulations could be made.

Metals, through oxidative mechanisms, may alter some cellular metabolic pathways, leading to the enhanced production of endogenous AhR ligands. The existence of endogenous AhR ligands has been suggested by numerous studies. The majority of these compounds, however, are weak inducers, which explains the relatively weak induction of Cyp1a1 mRNA by the metals when compared to TCDD. The first of these endogenous ligands are the heme degredation products bilirubin and biliverdin. Through the induction of HO-1, As^{3+}, Cd^{2+}, and Cr^{6+} may enhance the enzymatic conversion of heme to biliverdin and subsequently, bilirubin (Albores et al., 1989). Not only does bilirubin stimulate AhR transformation and DNA binding in vitro and in cell culture (Kapitulnik and Gonzalez, 1993), but it can also induce Cyp1a1 and a dioxin-response element in a dose and AhR-dependent manner (Phelan et al., 1998, Sinal and Bend, 1997). Biliverdin has also been shown to activate XRE-dependent gene transcription, although it may be an indirect effect, by serving as a metabolic precursor to bilirubin (Denison and Nagy, 2003). Arachidonic acid metabolites have also been identified as possible endogenous ligands of the AhR (Schaldach et al., 1999). Interestingly, oxidative stress has been implicated in the release of arachidonic acid from glycerophospholipids in cells (Tournier et al., 1997, Xu et al., 2003) which may suggest an increase in arachidonic acid metabolism in cells exposed to heavy metals.

Another postulation implicates posttranscriptional modifications of histones in the increase in gene transcription. As^{3+} induces phosphorylation (He et al., 2003, Li et al., 2001) and acetylation (Li et al., 2001) of histone H3 through the extracellular signal-regulated kinase (ERK), p38, and the Akt1 pathways. In accord, oxidative stress activates ERK (Aikawa et al., 1997), Akt (Ding et al., 2000), and p38 (Chuang et al., 2000). The phosphorylation-acetylation of histone H3 plays an important role in mediating the transcription of many genes by increasing the association of RNA polymerase II with target genes (Li et al., 2001). Recently, histone acetylation has been shown to be involved in the inducibility of Cyp1a1 mRNA transcription in human and mouse hepatoma cells (Shibazaki et al., 2004) and HeLa cells (Nakajima et al., 2003).

The above transcriptional mechanisms do not explain the effect of As^{3+}, Cd^{2+}, and Cr^{6+} on the inhibition of Cyp1a1 activity induction by TCDD. It is unlikely that translational mechanisms play a role in the reduction since Cyp1a1 protein is induced in Hepa 1c1c7 cells treated with these metals (chapter 2). However, oxidative mechanisms likely play a role in modifying Cyp1a1 function since NAC provided protection against a decrease in EROD activity while BSO potentiated the inhibition of EROD activity by the metals. One mechanism that has been offered to explain this phenomenon is the induction of HO-1. However, the induction of HO-1 does not explain why the weakest HO-1 inducer, Cr^{6+}, was the most potent at inhibiting TCDD-mediated induction of EROD activity. Furthermore, the addition of increasing concentrations of heme, which is also a potent inducer of HO-1, did not alter CYP450 expression while cotreatment of cells with As^{3+} and heme diminished CYP2B1/2 and CYP1A1 activities in rat hepatocyte cultures (Jacobs et al., 1999). Hence, although HO-1 induction may contribute to the inhibition of Cyp1a1 activity by the metals, there must be other interplaying mechanisms. Metal-induced ROS may oxidize thiol groups in cysteine residues, directly or indirectly through the formation of reactive nitrogen species, of the Cyp1a1 protein causing loss of protein function (Bogdan, 2001). ROS may also interact with the heme Fe^{2+} leading to heme destruction and enzyme inactivation (Lowe et al., 1998).

The cellular response to oxidative stress is activated to limit cellular damage caused by exposure to toxicants. With this, the diminution of Cyp1a1 activity decreases the rate of activation of AhR ligands into mutagenic species and limits further production of ROS. Likewise, it is anticipated that induction of Phase II enzymes may also be part of the cellular stress response since these enzymes protect the cell against the electrophilic by-products of the phase I metabolism of AhR ligands. Hence, the results of this study come at no surprise. We have not only confirmed that heavy metals induce both Nqo1 and Gst a at the transcriptional level, but we have also shown the role of oxidative stress in such modulation. BSO enhanced metal-induced expression of Nqo1 at the mRNA and activity levels and that of Gst a at the mRNA level. Meanwhile, NAC suppressed metal-induced expression of both genes.

The redox sensitivity of Nqo1 and Gst a transcription has been previously documented (Radjendirane et al., 1997, Tjalkens et al., 1998). It occurs through the antioxidant-response element (ARE) enhancer, located in the promoter region of the rat, mouse, and human genes. Multiple AREs, also called stress-response elements, are also found in promoter region of the mouse HO-1 gene. AREs bind to several nuclear transcription factors, including c-Jun, Jun-B, Jun-D, c-Fos, NF-E2-related nuclear factors (Nrf1, Nrf2), and the estrogen receptor (Jaiswal, 2004). Nrf2, a more potent activator of ARE than Nrf1, is retained in the cytoplasm by Keap1 which targets Nrf2 to proteasomal degradation. However, an increase in oxidative stress leads to the activation of protein kinase C (PKC) which phosphorylates Nrf2, leading to its release from Keap1. Nrf2 then translocates to the nucleus, dimerizes with c-Jun, Jun-B, and Jun-D, and binds to the ARE, leading to activation of Nqo1 and Gst a transcription (Jaiswal, 2004). Recent data have shown direct involvement of H_2O_2 in the activation of the Nrf2 system by As^{3+} (Pi et al., 2003).

Surprisingly, the presence of BSO significantly reduced Nqo1 activity, but further potentiated its mRNA expression in cells treated with As^{3+} with and without TCDD. The cause of this observation is unknown. However, it has been reported previously that Nqo1 expression is regulated by CYP1A1 activity (Marchand et al., 2004). A significant decrease in Cyp1a1 activity in the treatment group TCDD+As^{3+}+BSO is then paralleled by a decrease in Nqo1 activity. However, the regulation of Nqo1 by Cyp1a1 occurs at the transcriptional level and since Nqo1 mRNA was further potentiated in the presence of BSO, it is unlikely that a decrease in Cyp1a1 led to the inhibition of Nqo1 activity.

In the present study, we provide evidence for the role of oxidative stress in the modulation of AhR-regulated genes by heavy metals. As^{3+}-, Cd^{2+}-, or Cr^{6+}-mediated oxidative stress down-regulates Cyp1a1 activity posttranscriptionally but potentiates Nqo1 and Gst a expression at the transcriptional level. The mechanisms by which Cyp1a1 protein function is inhibited remain to be determined, but likely involve direct interactions with the heme protein. Also, the mechanisms by which oxidative stress mediates the transcriptional activation of Cyp1a1, Nqo1, and Gst a remain hypothetical and need to be further investigated. However, results derived from our study suggest that multiple signaling pathways and alterations in endogenous AhR-ligand production may be involved in the transcriptional activation of Cyp1a1. Also, the activation of transcription factors involved in the regulation of Nqo1 and Gst a genes may contribute to the transcriptional induction of these particular genes.

5.5.0.0. References

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6.0.0.0. Chapter 6.0

ASCORBIC ACID DIFFERENTIALLY MODULATES THE INDUCTION OF HEME OXYGENASE-1, NAD(P)H:QUINONE OXIDOREDUCTASE 1 AND GLUTATHIONE S-TRANSFERASE BY As^{3+}, Cd^{2+} AND Cr^{6+}.

A version of this chapter has been published: Elbekai, R.H., Duke, J. and El-Kadi, A.O., 2007. *Cancer Lett.*. **246**, pp. 54-62.

6.1.0.0. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that regulates the transcription of a multitude of genes, of which the most extensively studied are a number of drug metabolizing enzymes, including four phase I enzymes: cytochrome P4501a1 (Cyp1a1), Cyp1a2, Cyp1b1, and Cyp2s1; and four phase II enzymes: NAD(P)H: quinone oxidoreductase (Nqo1), glutathione *S*-transferase Ya subunit (Gst a), cytosolic aldehyde dehydrogenase and UDP-glucuronosyltransferase 1A6 (Nebert and Duffy, 1997, Rivera et al., 2002). Environmental contaminants, such as the halogenated aromatic hydrocarbons and nonhalogenated polycyclic aromatic hydrocarbons, are the most well characterized classes of AhR ligands (Denison and Nagy, 2003). The induced phase I drug metabolizing enzymes convert several of these AhR ligands into diol epoxides, which are responsible for mediating the carcinogenicity and mutagenicity of these environmental toxicants (Spink et al., 2002). The induction of the phase 2 enzymes, however, plays an important role in determining the cancer risk. Xenobiotic-induced carcinogenesis is hence regulated by a coordinated balance between the bioactivating phase I and the detoxifying phase 2 enzymes (Radjendirane et al., 1997).

Disruption of the coordinated regulation of the drug metabolizing enzymes will determine the mutagenicity of AhR ligands. The heavy metals As^{3+}, Cd^{2+}, and Cr^{6+}, which are environmental co-contaminants, inhibit AhR ligand-mediated induction of Cyp1a1 activity but potentiate the induction of Nqo1 and Gst a activities (chapter 2). Attempts to understand the mechanisms driving these responses, and thus define the exact role of AhR ligands and metals in carcinogenicity and mutagenicity, have found a role for oxidative stress in the modulation of AhR-regulated genes (chapter 5).

Interestingly, the induction of oxidative stress by heavy metals may also have therapeutic benefit. Arsenite, one of the most important environmental toxins, is associated with cancers of the skin, lungs, liver, kidney, and bladder. Yet despite its toxicity, As^{3+} has been used as a therapeutic agent for more than 2400 years. The efficacy of As^{3+} in the treatment of acute promyelocytic leukemia (APL) and multiple myeloma (MM) has been confirmed and further studies are investigating its role in the treatment of other malignancies (Hussein, 2001, Murgo, 2001). Various reports have linked the generation of reactive oxygen species (ROS) to the activity of As^{3+} in cancer (Jing et al., 1999). Hence, it could be assumed that antioxidants may alter the cytotoxicity of As^{3+}. Paradoxically however, AscA has been shown to enhance the anti-cancer effect of As^{3+} (Hussein, 2001). AscA enhanced the anti-lymphoma effect of As^{3+} without additional toxicity to normal tissue (Bachleitner-Hofmann et al., 2002). Also, AscA potentiated As^{3+}-mediated cell death of several myeloma cell lines and freshly isolated myeloma cells (Grad JM Blood 98, 2001).

With the proven role of Nqo1 and Gst a in the protection against certain types of cancers (Anttila et al., 1995, Katoh et al., 1995, Mulder et al., 1995, Rosvold et al., 1995), and the ability of AscA to potentiate the anticancer effect of arsenite, it is expected that this antioxidant will have a paradoxical effect on the ability of heavy metals, specifically arsenite, to induce AhR-regulated genes. In this study, we investigate the effect of AscA on the modulation of AhR-regulated genes by the three heavy metals As^{3+}, Cd^{2+}, and Cr^{6+}. These results were then compared to those obtained with the use of a prototype antioxidant, N-acetylcysteine (NAC).

6.2.0.0. Materials and Methods

6.2.1.0. Materials

Sodium arsenite, ascorbic acid, cadmium chloride, chromium trioxide, and N-acetylcysteine were purchased from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-tetrachlorodibenzo-p-dioxin, >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). TRIzol reagent, and the random primers DNA labeling system were purchased from Invitrogen Co. (Grand Island, NY). Hybond-N-nylon membranes were from Amersham Canada (Oakville, ON). [α-^{32}P]dCTP (3000 Ci/mmol) was supplied by the DNA Core Services Laboratory, University of Alberta. All other chemicals were purchased from Fisher Scientific (Toronto, ON).

6.2.2.0. Cell culture.

Hepa 1c1c7 cell lines, generously provided by Dr. O. Hankinson at the University of California (Los Angeles, CA), were maintained in Dulbecco's Modified Eagle's Medium (DMEM), without phenol red, supplemented with 10% heat-inactivated fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamycin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 cell culture flasks at 37°C in a 5% CO_2 humidified incubator.

6.2.3.0. Chemical treatments

Cells were treated in serum free medium alone or with 1 nM TCDD dissolved in DMSO, metals dissolved in double de-ionized water, or both. The various metals were added 30 min prior to the addition of TCDD, when applicable. TCDD was maintained in DMSO at -20°C until use but the metals were prepared freshly for each treatment. In all treatments, the concentration of double de-ionized water in treatment medium did not exceed 0.5% (v/v) while the DMSO concentration did not exceed 0.05% (v/v). When applicable, 1 mM AscA or 10 mM NAC, were added 1 or 6 h prior to addition of the metals, respectively.

6.2.4.0. RNA extraction and analysis

After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen Co). Northern blot analysis of total RNA was performed as described elsewhere (chapter 2).

The cDNA probes for mouse Cyp1a1, HO-1, and GAPDH mRNA were generously provided by Dr. John R. Bend (University of Western Ontario, London, ON). The cDNA probe for mouse Gst a mRNA was provided by Dr. David Eaton (University of Washington, Seattle, WA). The mouse QOR cDNA probe was prepared as previously described (Chen et al., 1994). All probes were [α^{32}P]-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions.

6.2.5.0. Determination of Intracellular As^{3+} levels

Instrumental neutron activation analysis (INAA) was used to determine the intracellular As^{3+} level of the study samples. Cellular homogenates were irradiated in batches in the University of Alberta SLOWPOKE II nuclear reactor for 2 h at a nominal thermal neutron flux of 5×10^{11} n cm^{-2} s^{-1}. Following a decay period of approximately 24 h the irradiated samples were individually counted for ~ 1 h at a sample-to-detector distance of 1 cm. All counting was performed using a 22 % relative efficiency ORTEC hyperpure Ge detector (FWHM of 1.95 keV for the 1332.5 keV full energy peak of ^{60}Co) housed in a 10 cm Pb cave. Elemental analysis was performed by the semi-absolute method of activation analysis. As^{3+} was quantified using the 559.1 keV gamma-ray emission of ^{76}As ($t_{1/2}$ = 1.0778 d) produced *via* the neutron reaction $^{75}As(n,\gamma)^{76}As$. SCP Science NIST traceable PlasmaCal standards (9.97 µg/mL and 100.9 µg/mL As) were used for calibration purposes.

6.2.6.0. Statistical analysis

All results are presented as mean ± S.E. The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc comparison. The differences were considered significant when $P<0.05$.

6.3.0.0. Results

6.3.1.0. Ascorbic acid: pro-oxidant properties?

The induction of heme oxygenase-1 (HO-1), which is considered to be an adaptive cellular mechanism in response to stressful stimuli, was used as a marker of oxidative stress (Poss and Tonegawa, 1997). As expected, As^{3+}, Cd^{2+}, or Cr^{6+} alone (Figure 6.1A) or co-treated with TCDD (Figure 6.2A) significantly induced HO-1 mRNA levels, although to varying degrees. As^{3+} was the most potent inducer of HO-1 mRNA (1500% increase) while Cd^{2+} was the second most potent and Cr^{6+} the least potent. With what is known about the induction of HO-1, it was not surprising to see a significant inhibition in the induction of this gene by all metals when the cells were pretreated with the antioxidant NAC (Figure 6.1A and 6.2A). Similarly, AscA also significantly inhibited the induction of HO-1 mRNA by Cd^{2+} and Cr^{6+}. Inconsistent with its antioxidant properties however, AscA potentiated the induction of HO-1 mRNA by As^{3+} in the absence (Figure 6.1A) and presence of TCDD (Figure 6.2A), although this effect was most pronounced in the presence of TCDD.

6.3.2.0. AscA modulates Nqo1 and Gst a mRNA levels

As^{3+} or Cd^{2+} alone significantly induced Nqo1 and Gst a mRNA levels (Figure 6.1) and potentiated their induction by TCDD (Figure 6.2B). Cr^{6+} however had no significant effect on Nqo1 or Gst a mRNA levels (Figures 6.1B and 6.2B). In parallel to the HO-1 mRNA results, NAC diminished metal-mediated induction of Nqo1 and Gst a mRNA levels, at the constitutive level (Figure 6.1B). The most significant decrease was in cells treated with As^{3+} as there was a 1.5 and 1.7 fold decrease in Nqo1 and Gst a mRNA, respectively, in cells treated with As^{3+}+NAC compared to cells treated with As^{3+} alone (Figure 6.1B). Surprisingly, while having no effect on its own, AscA superinduced the induction of Nqo1 and Gst a mRNA by As^{3+} in the absence and presence of TCDD, while inhibiting the induction by Cd^{2+} and Cr^{6+}. Thus, it seems that AscA modulates HO-1, Nqo1 and Gst a gene expression by similar mechanisms.

6.3.3.0. AscA differentially modulates Cyp1a1 mRNA expression

To understand how AscA alters the balance in the expression of phase I and phase II enzymes, we measured the effect of AscA pretreatment on the expression of Cyp1a1 mRNA at the constitutive and inducible levels. As^{3+} and Cr^{6+} up-regulated the expression of Cyp1a1 mRNA, alone (Figure 6.1C), and As^{3+} further potentiated the induction by TCDD (Figure 6.2C). NAC diminished the induction of Cyp1a1 mRNA levels by As^{3+} or Cr^{6+} alone, and inhibited As^{3+}-mediated potentiation of the mRNA levels in the presence of TCDD (Figure 6.2C). However, AscA significantly inhibited the constitutive induction of Cyp1a1 mRNA by the three metals (Figure 6.1C) while further potentiating the induction of mRNA levels in cells treated with TCDD and metals (Figure 6.2C).

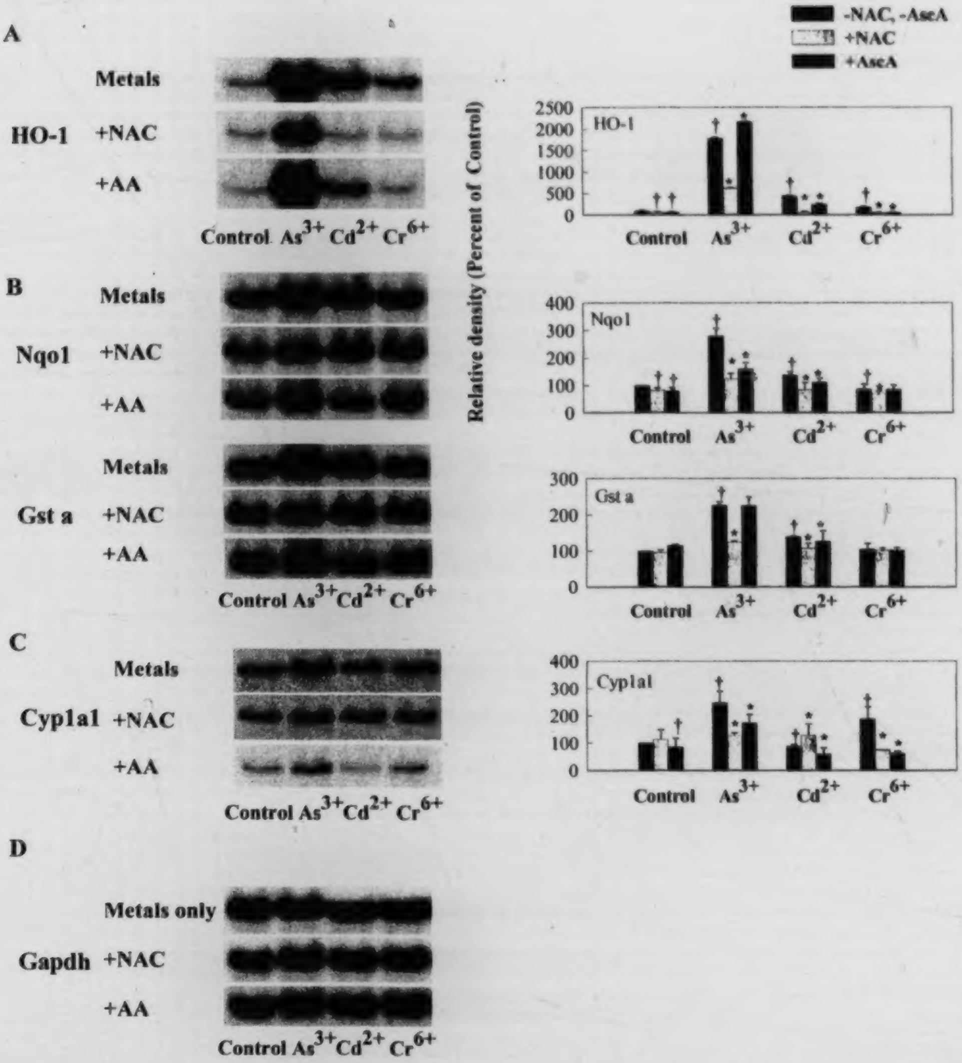

Figure 6.1. The effect of NAC and AscA pretreatment on heavy metal-mediated inductions of HO-1 (A), Nqo1 and Gst a (B), and Cyp1a1 (C) mRNA in Hepa 1c1c7 cells. Cells were exposed to 5 μM of As^{3+}, Cd^{2+}, or Cr^{6+} for 6 h. When applicable, cells were pretreated with 10 mM NAC or 1 mM. Total RNA was extracted, separated on a denaturing gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled cDNA probe. Gapdh was used as a loading control (C). The graphs demonstrate mRNA levels, as quantified by densitometry. $^{\dagger}p < 0.05$, compared to control; $^{+}p < 0.05$, compared to TCDD only; $*p < 0.05$, +NAC/+AscA treatments compared to respective –NAC/-AscA treatments(n=3).

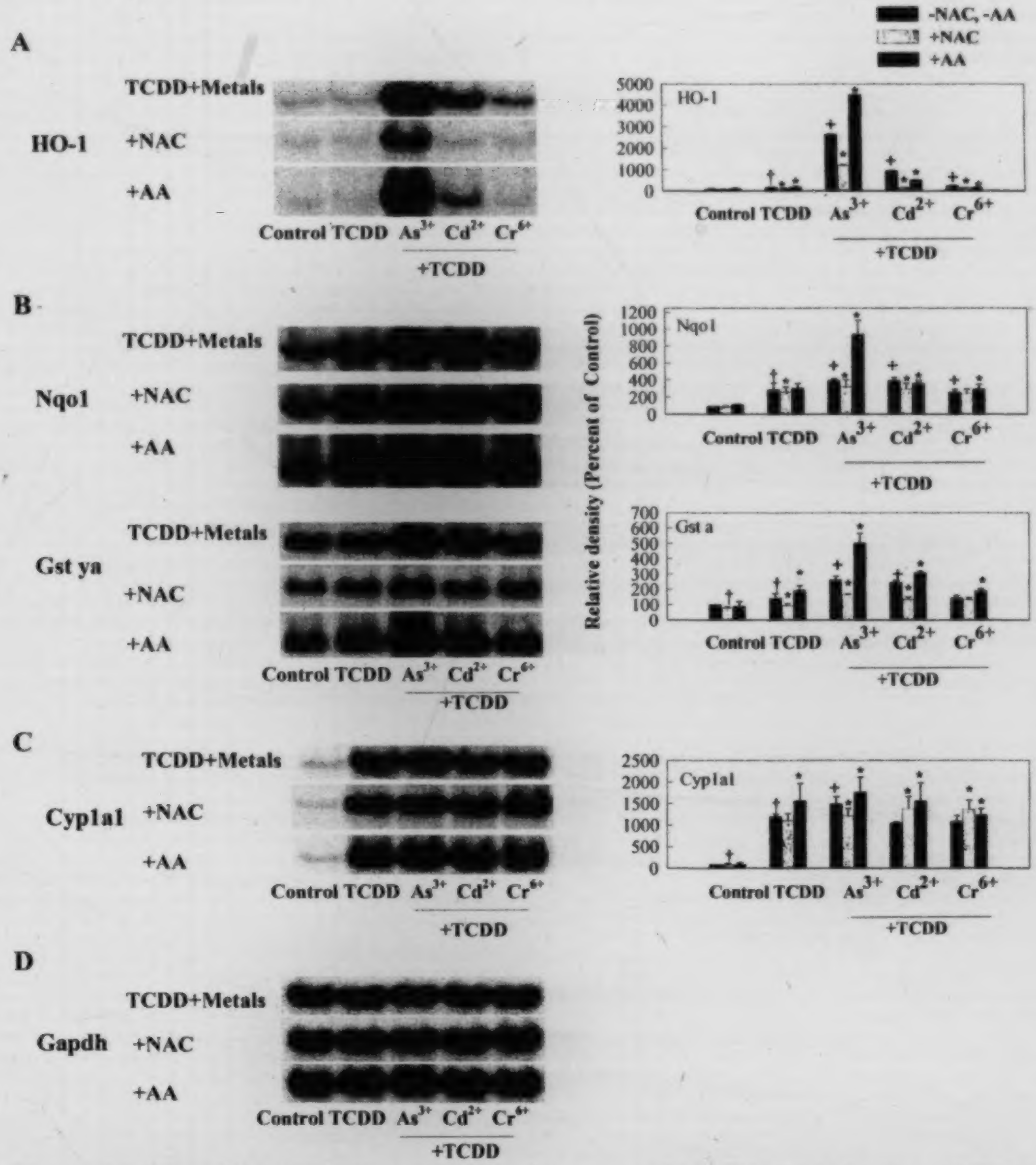

Figure 6.2. The effect of NAC and AscA on heavy metal-mediated induction of HO-1 (A), Nqo1 and Gst a (B), and Cyp1a1 (C) mRNA in Hepa 1c1c7 cells in the presence of TCDD. Cells were exposed to 1 nM TCDD with 5 μM of As^{3+}, Cd^{2+}, or Cr^{6+} for 6 h. When appkicable, cells were pretreated with 10 mM NAC or 1 mM AscA. Total RNA was extracted, separated on a denaturing gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled cDNA probe. Gapdh was used as a loading control (C). The graphs demonstrate mRNA levels, as quantified by densitometry. $^{\dagger}p < 0.05$, compared to control; $^{*}p < 0.05$, +NAC/+AscA treatments compared to respective –NAC/-AscA treatments (*n*=3).

6.3.4.0. Effect of ascorbic acid on the cellular uptake and efflux of As^{3+}

Using INAA, intracellular As^{3+} was determined in cells treated with As^{3+} in the presence and absence of TCDD with and without NAC/AA pre-treatment (Fig. 6.3). Surprisingly, NAC significantly decreased the intacellular As^{3+} concentration in cells treated with As^{3+} in the absence and presence of TCDD. On the other hand, although AA slightly decreased the intracellular As^{3+} content in cells treated with metal alone, it did not alter its concentration in cells treated with As^{3+} and TCDD (Fig. 6.3). Thus, AA modulates the effect of As^{3+} on AhR-regulated genes without altering its cellular influx of efflux.

6.4.0.0. Discussion

Nqo1 and Gst a are key enzymes involved in cellular defence against reactive forms of oxygen and the inhibition of carcinogenesis. Among many roles, these enzymes are involved in the detoxification of highly reactive species, such as quinones (Nioi and Hayes, 2004). Genetic polymorphisms in the Gst multigene family have been associated with a higher level of carcinogen-DNA adducts, more cytogenetic damage, and an increased risk in the development of lung, bladder, and prostate cancer, and ALL (acute lymphocytic leukemia) (Agalliu et al., 2005, Srivastava et al., 2005, Ye and Song, 2005). Also, an inactivating polymorphism in Nqo1 confers susceptibility to infant ALL and AML (acute myelogenous leukemia) and several other types of cancers (Rosvold et al., 1995, Traver et al., 1992). The heavy metals As^{3+}, Cd^{2+}, and Cr^{6+} produce a state of oxidative stress that is responsible for the induction of HO-1, Nqo1 and Gst a at the transcriptional level (chapter 5). In view of the importance of these enzymes in carcinogenicity, and the rising significance of As^{3+} and the antioxidant AscA in the treatment of APL, it was of interest to examine the effect of AscA on the induction of these genes by the heavy metals.

In this study, we initially confirmed the role of oxidative stress in the induction of HO-1, Nqo1 and Gst a by As^{3+}, Cd^{2+}, and Cr^{6+} by pretreating the cells with the antioxidant NAC. NAC suppressed metal-induced expression of these genes by the three metals. Surprisingly however, although AscA ameliorated the induction of these genes by Cd^{2+}

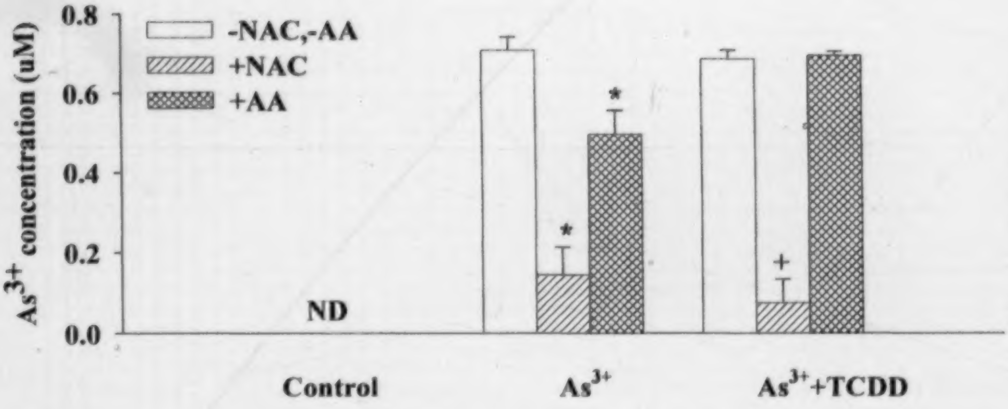

Figure 6.3. The effect of NAC and AA on intracellular As^{3+} concentration in Hepa 1c1c7. INAA was used to determine the intracellular As^{3+} concentration using cellular homogenates. Samples were irradiated in a nuclear reactor and subsequently counted using an ORTEC hyperpure Ge detector. As^{3+} was quantified using the 559.1 keV gamma-ray emission of ^{76}As (t½ = 1.0778 d) produced *via* the neutron reaction ^{75}As(n,γ)^{76}As. Data are expressed as mean concentration ± SE where *n*=4. **p*<0.05 compared to As^{3+} alone; +*p*<0.05 compared to As^{3+}+TCDD. ND=not detectable.

and Cr^{6+}, it potentiated the mRNA induction by As^{3+}. These results, although surprising, support the combination of AscA and As^{3+} in the management of cancer.The induction of redox sensitive genes by AscA had been previously reported. AscA increased HO-1 mRNA expression in both AGS and KATO III cells (Becker et al., 2003). Also, AscA increased NQO1 activity in a dose-dependent manner in Colo205 colon cancer cells (Wang and Higuchi, 1995) and UDP glucuronyl transferase in adult guinea pig livers (Roomi et al., 1998).

Thus, although the ability of AscA to induce phase II enzymes has been clearly established, the mechanism by which it acts synergistically with As^{3+} in the treatment of cancer is still controversial. The mechanisms by which As^{3+} itself induces remission in APL and MM are not completely understood but the generation of ROS is in no doubt a significant contributor to its cytotoxicity. Hence, it is paradoxical that AscA enhances the therapeutic efficacy of this heavy metal. It has been speculated that the pro-oxidant properties of AscA, observed in vitro, could increase the state of oxidative stress induced by As^{3+} and hence enhance the cytoxicity of As^{3+}. The pro-oxidant activity of AscA in vitro is a well documented phenomenon that is dependent on the reduction of free transition metal ions (Noto et al., 1989). However, with our data showing an inhibition in Cd^{2+}- and Cr^{6+}- induced increase in HO-1, Nqo1 and Gst a mRNA expression, AscA is undoubtedly functioning as a strong antioxidant in Hepa 1c1c7 cells. It has already been postulated that HO-1 is induced by stressful and non-stressful stimuli (Immenschuh and Ramadori, 2000). Hence, AscA is a non-stressful stimulus of HO-1, Gst a, and Nqo1 in the presence of As^{3+}. Taking into account the role of these enzymes in the protection against carcinogenesis, a combination of AscA and As^{3+} is an interesting option in the treatment of APL.

The mechanism by which AscA potentiates the effect of As^{3+} on the expression of HO-1, Nqo1 and Gst a is difficult to ascertain at this point with the results available and what is known about the regulation of these enzymes. Some assumption may, however, be made. The primary determinant of cellular sensitivity to As^{3+} is intracellular glutathione (GSH) levels (Yang et al., 1999). Cells expressing higher levels of GSH are less sensitive to As^{3+} (Lee et al., 1989). This is not only due to the antioxidant properties of GSH, but also to the direct interaction of GSH with As^{3+}. GSH complexes with As^{3+} by a physical interaction (Scott et al., 1993). Therefore, agents which decrease intracellular GSH levels will synergistically potentiate the anticancer effect of As^{3+} by increasing the levels of free As^{3+} in the cell. Interestingly, AscA depleted GSH in mononuclear cells from the bone marrow in patients with MM (Grad et al., 2001). This, however, is unlikely the case as it is clear from our results that AscA does not alter the intracellular As^{3+} concentration (Figure 6.3). It is likely though that signaling pathways are involved in the modulation of AhR-regulated genes by AscA.

Modulation of HO-1, Nqo1 and Gst a transcription occurs through the ARE enhancer, located in the promoter region of the rat, mouse, and human genes (Radjendirane et al., 1997, Tjalkens et al., 1998). Multiple AREs are also found in promoter region of the mouse HO-1 gene. The Nqo1 gene ARE contains one perfect and one imperfect AP-1 elements (Jaiswal, 2000). The same holds true for other gene AREs. AP-1 is a complex protein composed of *jun* and *fos* homodimers and heterodimers. AscA has been shown to increase AP-1 binding to DNA and to increase the mRNA levels of c-jun, junB, and c-fos in 1 alpha,25(OH)2D3-induced cells (Lopez-Lluch et al., 2001), likely by activating the ERK MAP kinase pathway (Park et al., 2005). Activated ERK can phosphorylate several cytosolic and nuclear proteins, leading to the induction of AP-1 and the activation of AP-1-dependent genes (Yu et al., 1999).

In conclusion, HO-1, Nqo1, and Gst a mRNA induction by As^{3+}, but not Cd^{2+} or Cr^{6+}, is enhanced by AscA. It is therefore tempting to speculate that this mechanism plays an important role in enhancing the anticancer effect of As^{3+} observed in patients with APL. AscA may potentiate the therapeutic efficacy of As^{3+} by enhancing the expression of HO-1, Nqo1, and Gst a while acting as a potent antioxidant and inhibiting Cyp 1a1 mRNA induction. Thus, these results suggest that AscA may increase the antineoplastic effect of As^{3+} while providing protection to normal cells. Further studies are needed to enhance our understanding of the mechanisms by which AscA potentiates the effect of As^{3+} on Nqo1 and Gst a expression. These studies may lead to the development of treatment regimens directed at enhancing the synergy between As^{3+} and many other possible agents.

6.5.0.0. References

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7.0.0.0. Chapter 7.0

GENERAL DISCUSSION AND CONCLUSIONS

7.0.0.0. General Discussion and Conclusions

Heavy metals and AhR ligands are carcinogenic environmental co-contaminants. Quantitatively, the extent of human exposure to mixtures of AhR ligands and heavy metals in not known. However, these complex mixtures may exert a biological effect that is different due to possible synergistic or antagonistic interactions by multiple mechanisms. With what is known about the toxicity of AhR ligands, their toxicity may be modified by two main mechanisms: competition for their sites of action or metabolizing enzymes, or by influencing their metabolism by the various metabolic activating or detoxifying enzymes. As such, we undertook extensive studies to investigate the effect of metals on the modulation of the AhR-regulated genes: Cyp1a1 and Nqo1. In our first study, we report the ability of As^{3+}, Cd^{2+}, and Cr^{6+} to influence the bioactivation of AhR ligands by phase I drug metabolizing enzymes and the inactivation of their reactive intermediates by phase II drug metabolizing enzymes. In the second and third studies, to decipher the molecular mechanisms involved in the alteration of Cyp1a1 and Nqo1 expression, we analyzed the effect of As^{3+}, Cd^{2+}, and Cr^{6+} on each step of the AhR-signaling pathway. Thus, we examined the effect of heavy metals on the transcriptional activation of Cyp1a1 and Nqo1, and posttranscriptional and posttranslational modifications of the gene products. Lastly, we examined the role of metal-induced oxidative stress on the expression of these AhR-regulated genes.

In all studies, all three metals increased Cyp1a1 mRNA levels. As^{3+}, Cd^{2+}, and Cr^{6+} induced constitutive mRNA expression and potentiated the induction of Cyp1a1 mRNA by TCDD, 3-MC, βNF, and BaP. On the other hand, although Cr^{6+} induced constitutive Cyp1a1 mRNA and potentiated 3-MC-, βNF-, and BaP-mediated induction of Cyp1a1 mRNA levels, it significantly inhibited the time-dependent induction of Cyp1a1 mRNA by TCDD at 3, 12, and 24 h, but potentiated the induction at 6 h. Previous work investigating the effect of heavy metals on Cyp1a1 mRNA has produced conflicting results. In human HepG2 cells, As^{3+} has been reported to either have no effect, or to decrease AhR ligand-dependent increase in CYP1A1 mRNA (Bonzo et al., 2005, Vakharia et al., 2001, Wu et al., 2003). Similarly, in human breast cancer cells (T-47-D), the same group reported a potentiation in BaP-mediated induction of CYP1A1 mRNA by As^{3+} (Spink et al., 2002) and later published results indicating that As^{3+} did not alter BaP-induced CYP1A1 mRNA levels in the same cell line (Wu et al., 2003). Yet in the human lung adenocarcinoma cell line (Ho and Lee, 2002), As^{3+} potentiated BaP-induced CYP1A1 mRNA and increased CYP1A1 mRNA in the rat lung (Seubert et al., 2002). The results we have demonstrated in the Hepa 1c1c7 cells however have been confirmed by Kann et al. (Kann et al., 2005). Thus, it appears that the modulation of Cyp1a1 mRNA levels by heavy metals is both a species and tissue-dependent effect.

We determined that all three metals increase Cyp1a1 mRNA by activating gene transcription and increasing mRNA stability. Furthermore, it is apparent that oxidative stress plays a role in the increase in Cyp1a1 mRNA levels. Transcriptional activation of the *Cyp1a1* gene proceeds through activation of the AhR, in line with the data demonstrating the ability of CHX and the proteasome inhibitor MG-132 to potentiate metal-induced Cyp1a1 mRNA (chapter 3). It is believed that CHX potentiates the induction of Cyp1a1 mRNA by TCDD by inhibiting the synthesis of the AhR degradation promoting factor, a labile protein with possible ubiquitin-ligase activity, thus prolonging the effect of the AhR (Roberts and Whitelaw, 1999, Santiago-Josefat et al., 2001).

EMSA and luciferase experiments confirmed the role of the AhR in metal-mediated Cyp1a1 mRNA induction. Interestingly, the EMSA experiements revealed a possible ligand-independent mechanism of AhR activation. Cd^{2+} and Cr^{6+}, but not As^{3+}, induced nuclear accumulation of the AhR and AhR-DNA binding but did not induce AhR transformation *in vitro* (chapter 3). Thus, the metals themselves are not AhR ligands, but activate the AhR through an indirect mechanism, through the induction of oxidative stress.

Oxidative stress may mediate the transcriptional activation of the *Cyp1a1* gene through the production of endogenous ligands. All three metals induce HO-1 levels and decrease heme content, indicating an increase in the production of heme degradation products, which may in turn stimulate AhR transformation and DNA binding (Kapitulnik and

Gonzalez, 1993). Oxidative stress-mediated release of AA from glycerophospholipids may result in increased production of AA metabolites, which have also been identified as possible endogenous ligands of the AhR (Schaldach et al., 1999, Tournier et al., 1997, Xu et al., 2003).

Activation of the AhR may also be a consequence of ROS-induced activation of multiple signaling pathways. PKC isoenzymes are activated by ROS through their C1 domain, although the exact isoforms responsive to activation by oxidative stress are not known (Barnett et al., 2007). Interestingly, PKC-dependent phosphorylation of the AhR and ARNT is required for transcriptional activity of the AhR-ARNT complex (Hankinson, 1995). However, the exact role of PKC-mediated induction of AhR transformation by heavy metals remains to be determined.

Although the mechanism of metal-mediated increase in Cyp1a1 mRNA has been identified to be AhR-dependent, the mechanism of Cyp1a1 mRNA stabilization by the heavy metals is still in question. mRNA decay is a regulated process through AU rich elements, in the 3′ untranslated region, that bind AU binding proteins (AUBPs), which govern their stability (Gingerich et al., 2004). Activation of AUBPs by cellular signals occurs by multiple mechanisms, including changes in their phosphorylation or ubiquitination status. The regulated degradation of Cyp1a1 mRNA has not been elucidated and as such, it is difficult to ascertain the exact mechanism of metal-mediated mRNA stabilization, although it is likely due to the activation of AUBPs that bind the Cyp1a1 mRNA transcripts and enhance their stability. hnRNP A1 is an AUBP that binds the AU rich element in Cyp2a5 mRNA, prolonging the half life of the transcripts (Aida and Negishi, 1991, Geneste et al., 1996, Glisovic et al., 2003). Interestingly, increased binding of hnRNP A1 to mRNA is important in ensuring cell survival during the stress response, such as that seen in arsenite-stressed cells (Guil et al., 2006). As^{3+} also induces the formation of stress granules containing Hu antigen R, another AUBP involved in stabilizing mRNA (David et al., 2007). More research is needed to verify the role of AUBPs in Cyp1a1 mRNA stabilization by heavy metals.

The paradoxical decrease in Cyp1a1 activity despite the increase in Cyp1a1 mRNA dictates the presence of posttranscriptional mechanisms involved in inhibiting Cyp1a1 protein activity. None of the three metals however decrease Cyp1a1 protein levels, but rather increase the stability of the synthesized protein (chapter 3). Thus, it is apparent that metals inhibit Cyp1a1 protein function, through direct or indirect mechanisms. In either case, the production of ROS is a central mediator in the down-regulation of Cyp1a1 activity. ROS may directly interfere with Cyp450 protein function by oxidizing thiol groups in critical cysteine residues, causing changes in protein conformation and interfering with the coordination of the heme prosthetic group (Bogdan, 2001). Indirectly, ROS may also modulate Cyp450 protein function by increasing the degradation of heme by HO-1. In fact, Cyp450 heme has been shown to be a good substrate of HO in the liver (Kutty et al., 1988).

The decrease in Cyp1a1 activity and the increase in Nqo1 activity by heavy metals appear to be part of a tightly regulated sequence of events that are part of a cellular stress response to limit cell damage. As^{3+} and Cd^{2+} activate Nqo1 transcription, which is perceived as an increase in mRNA and activity levels. As with the mechanism involving Cyp1a1 mRNA induction, the induction in Nqo1 was also confirmed to be mediated through oxidative stress. On the contrary, the induction of Nqo1 is dependent on *de novo* protein synthesis of a labile protein as CHX inhibited the induction of Nqo1 by the metals. Furthermore, unlike AhR ligands, the AhR is not a central mediator of metal effects on the phase II enzymes as As^{3+} and Cd^{2+} induced Nqo1 activity in AhR deficient Hepa 1c1c7 cells (chapter 1). This information implicates the involvement of Nrf2 and the ARE, and not the XRE.

Not surprisingly, arsenic activated Nrf2 in osteoblasts (Aono et al., 2003) and cultured keratinocytes (Pi et al., 2003) and induced Nqo1 by disrupting the Nrf2-Keap1 complex (He et al., 2006). Similarly, Cd^{2+} induced HO-1 expression, paralleled by an increase in the half-life of Nrf2 (Stewart et al., 2003).

Metal-mediated increase in Nrf2 levels is likely to be mediated by the induction of oxidative stress. The level of cellular Nrf2 at any time point is regulated by a balance between its rate of synthesis and its rate of degradation by the proteasome. Under conditions of oxidative stress, Nrf2 is liberated from its negative regulator, Keap1, and hence the rate of Nrf2 degradation is reduced, favoring the expression of Nrf2 (Jaiswal, 2004, Ma et al., 2004, McMahon et al., 2001, Nioi and Hayes, 2004). Liberation of Nrf2 from Keap1 interaction occurs during times of oxidative stress through multiple signaling pathways, through direct and indirect mechanisms. Modification of the Keap1 protein through oxidation of its reactive cysteines by ROS may dampen its inhibitor effect on Nrf2, although this mechanism alone may be insufficient for liberation of Nrf2 (Eggler et al., 2005, Wakabayashi et al., 2004, Zhang and Hannink, 2003). Activation of phosphorylation pathways is required for the liberation of Nrf2 and its subsequent translocation to the nucleus. The phosphorylation activities of PKC (Huang et al., 2000), MAPKs (Yu et al., 2000), and PI3K (Kang et al., 2002) have been implicated in the phosphorylation of Nrf2, resulting in its release from Keap1, although no specific phosphorylation targets for the latter two have been identified. As discussed earlier, oxidative stress-mediated activation of PKC occurs through modification of the C1 domain by ROS. Similarly, the small GTPase Ras, which is the the upstream regulator of all the MAPKs and PI3K, is activated by ROS as a result of the modification of a cysteine residue close to the guanine nucleotide binding site (Torres, 2003).

Contrary to the effects of As^{3+} and Cd^{2+}, Cr^{6+} inhibited constitutive, and to a greater degree, inducible Nqo1 expression. One of the mechanisms by which Cr^{6+} may decrease Nqo1 mRNA is by increasing the rate of mRNA degradation as Cr^{6+} decreased the Nqo1 mRNA by more than half. The mechanisms by which Cr^{6+} inhibits phase II enzyme expression may be multiple and various. One of these mechanisms involves an increase in the degradation of the Nqo1 mRNA transcripts as Cr^{6+} decreased the Nqo1 mRNA half-life to more than half (chapter 4). The mechanisms involved in Nqo1 mRNA degradation have yet to be studied. Information provided here however provides evidence for a regulated degradation process. Thus, it is likely that Cr^{6+} induces binding of AUBPs to Nqo1 mRNA that promote destabilization and consequent degradation of the mRNA transcripts.

The decrease in Nqo1 mRNA stability is an inadequate explanation for why the decrease in Nqo1 expression is more apparent in the presence of AhR ligands. This distinction likely occurs at the transcriptional level. The inhibition of inducible gene expression has been confirmed by Wei et al., who have demonstrated the ability of Cr^{6+} to interfere with the binding of the coactivator p300, and associated histone acetylation, to chromatin (Wei et al., 2004).

Thus, As^{3+} and Cd^{2+} increase Nqo1 activity by inducing Nqo1 transcription through a ROS-dependent mechanism. From our results, it is apparent that the Nqo1 protein is not as sensitive as Cyp1a1 to ROS-mediated protein degradation as an increase in mRNA by heavy metals is directly translated to an increase in protein activity. However, the inhibition of Nqo1 activity, but not mRNA, in cells treated with As^{3+} and BSO dictates the inhibition of Nqo1 protein activity under conditions of excessive ROS production (Figure 5.5).

In chapter 5.0, our use of NAC confirmed the role of ROS in the modulation of Cyp1a1 and Nqo1 expression. NAC has been extensively used in the literature to dampen metal-induced ROS levels in the cells and determine the role of oxidative stress in the modulation of cellular processes by heavy metals (Barchowsky et al., 1996, Roybal et al., 2005, Ryabinina et al., 2006). However, we later demonstrated a decrease in intracellular As^{3+} levels in cells pretreated with NAC, likely due to direct chelation of the metal ions and subsequent cellular export by ATP-dependent transporters, such as P-glycoprotein (Liu et al., 2001). At this point, we cannot determine whether the observations made in our studies are a result of the antioxidant, or the chelating properties of NAC. However, the data obtained from cells pretreated with BSO confirms the involvement of ROS in the modulation of Nqo1 and Cyp1a1 expression (chapter 5.0). Furthermore, AscA inhibited the induction of constitutive Cyp1a1 and Nqo1 expression by the heavy metals, verifying the role of oxidative stress for the upregulation of AhR-regulated genes at the transcriptional level (Figure 6.1).

More information is needed to determine the exact redox-sensitive signaling pathways, as well as the down-stream effectors, involved in the modulation of Cyp1a1 and Nqo1 by the heavy metals. Previous work has documented the role of PKC and MAPKs in modulating AHR/ARNT–dependent gene expression (Hankinson, 1995, Nioi and Hayes, 2004, Tan et al., 2004). Interestingly, although these kinases exert their effect on the AhR by direct phosphorylation of effector proteins in the AhR or Nrf2 signal transduction pathways, other downstream mediators of PKC have been implicated in modulating AhR-regulated gene expression. AhR ligands induce the expression of c-Fos, c-Jun, JunB, and JunD, and increase AP-1 binding, under the direction of PKC (Hoffer et al., 1996). As^{3+} and Cd^{2+} have also been shown to activate gene transcription through AP-1 dependent mechanisms (Elbirt et al., 1998, Henson and Chedrese, 2004). It has been hypothesized that activation of PKC by AhR ligands facilitates expression of AhR-regulated genes through activation of AP-1 activity (Chen and Tukey, 1996). Interstingly, AP-1 binds to the Cyp1a1 promoter region and induces Cyp1a1 mRNA (Carriere et al., 1998). Thus, the role of AP-1 in metal-mediated induction of Cyp1a1 must be closely examined. NF-kB, another redox-sensitive transcription factor, has been shown to suppress AhR-activated genes (Ke et al., 2001). In parallel, RelB, a subunit of NF-kB, physically interacts with the AhR and increases TCDD-induced XRE reporter gene activity (Vogel et al., 2007). Interstingly, As^{3+}, Cd^{2+} and Cr^{6+} inhibited NF-kB binding to DNA *in vitro*, offering an alternate explanation for the potentaition in TCDD-mediated induction of AhR-regulated genes (Shumilla et al., 1998).

Although the effect of metals on the metabolism of xenobiotics has not been tested, it would seem that the combination of metals and AhR ligands causes an imbalance in the regulation of phase I and II drug metabolizing enzymes. Thus, there is a need to evaluate the biological response of metal–AhR ligand combinations as it would seem that the combination confers a response that is different than expected based on the toxicological mechanisms of each class evaluated separately. As^{3+} and Cd^{2+} may decrease the carcinogenic and mutagenic effects of AhR while the effect of Cr^{6+} is not clear as it reduced the activities of both Cyp1a1 and Nqo1.

The therapeutic benefits of As^{3+} in treating various malignancies is proof to the hypothesis that the combination of AhR ligands and heavy metals imposes a biological and toxicological response that is different than is expected, based on the mechanisms of toxicity of each class evaluated separately. In fact, based on the evidence provided, it is tempting to speculate that modulation of AhR-regulated genes by As^{3+} plays a role in its clinically observed anticancer effect. Future studies are thus required to determine the effect of heavy metals on the metabolism of AhR ligands to quantify their effect on the mutagenicity of environmental pollutants and determine the exact role of AhR-regulated genes in the initiation and progression of malignancies.

7.1.0.0. Future objectives:

(1) To determine the effect of co-exposure to heavy metals and AhR ligands on the expression of AhR-regulated genes *in vivo*,

(2) To characterize the role of NF-kB and AP-1 signalling pathways in the modulation of AhR-regulated genes by heavy metals,

(3) To determine the role of co-activator and co-repressor proteins in the interactions between heavy metals, the AhR and AP-1 and/or NF-kB signalling pathways, and

(4) To identify sets of genes that mediate the cross-talk between the AhR and AP-1 and/or NF-kB.

7.2.0.0. References

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